Epidemiology and Pathogenesis of Paratuberculosis

Philippa M. Beard

PhD

The University of Edinburgh

2000

Dedication

For Mum and Dad, for all their support, love and patience.

Declaration

I declare that this thesis has been composed entirely by myself and that the work contained in it, except where clearly stated, was performed by myself.

19 Feb 01

Acknowledgements

The preparation of inoculum, retrospective counts, culture of samples of tissue, faeces and urine and the detection of IS900 was carried out by Amanda Pirie, Karen Rudge and Dennis Henderson. The FACS analysis described in chapter 6 was carried out by Louise Wildblood, Catherine Sinclair and Alan Ross. The preparation of histological slides was performed by Valerie Forbes and Alison Baird. Mike J. Daniels collected the wildlife samples in survey 4.2. Neil MacIntyre, Andrew Dawson and Sharon Moss performed the Schmorls stain described in section 2.1.8. Iain McKendrick provided statistical support, especially for the analyses in chapter 6. The study descibed in section 4.4 was funded by a grant from the British Wildlife Health Association.

My supervisors Dave Buxton, Susan Rhind and Mike Sharp have provided endless support, encouragement and advice, for which I am eternally grateful. I have benefited from assistance of the pathology departments of Moredun Research Institute, Central Veterinary Laboratories at Lasswade, and the Easter Bush Veterinary Centre. I would particularly like to acknowledge the assistance of Alex Schock, Lorenzo Gonzales, Stephen Maley, Ranold Munro, Sandra Scholes, Martin Jeffrey, Robert Higgins, Alastair Wood, Cheryl Scudamore and Isabel Begara-McGorum.

I thank Jock McCracken, Colin MacAldowie, Manus Graham, Iris Campbell and all the stockmen and women for their help with the animal work, Matthew Gilmour and Brian Easter for advice on the production of the photographic plates, Diane Donaldson and Heather Edgar for accessing obscure references, Christine Curran for advice on the intricacies of Microsoft Word, and the ladies in the refectory for a regular supply of puddings with custard, even when I was late.

Finally, my thanks go to the colleagues who helped me during the three years of this PhD – Kym Abbott, Richard Whittington, Mike J. Daniels, Alastair Greig, Sandy Clark, Karen Stevenson, Chris Cousens, Chris Summers, Karen Walsh, Kath Thomas, Rubina Sharif and Val Hughes, to name but a few.

Contents

Abstract

Paratuberculosis is a common and fatal disease of ruminants, caused by the bacterium *Mycobacterium avium* subsp. *paratuberculosis* (*M.a. paratuberculosis*). The disease causes significant economic and welfare concerns for affected farms and ruminant livestock industries worldwide, however, the pathogenesis and epidemiology of paratuberculosis are poorly understood, resulting in few successful strategies available for control or prevention programmes. This thesis describes investigations into three aspects of the epidemiology and pathogenesis of paratuberculosis - the extent to which non-ruminant wildlife are infected with *M.a. paratuberculosis*, the initial host response to *M.a. paratuberculosis* exposure, and, finally, the influence of host genotype on susceptibility to the disease.

In response to the recent discovery of natural *M.a. paratuberculosis* infection of non-ruminant wildlife in Scotland, the pathology of natural rabbit paratuberculosis was investigated and described. Infected rabbits exhibited mild or severe histopathological lesions in the gastrointestinal tract and associated lymphoid tissues. *M.a. paratuberculosis* was cultured from both the faeces and urine of naturally infected rabbits, revealing two possible routes of transmission of the organism.

To investigate the impact of natural rabbit paratuberculosis on the disease in ruminant livestock, young calves were orally inoculated with an isolate of *M.a. paratuberculosis* from a naturally infected rabbit. After an incubation period of six months, the organism was recovered from the intestinal tissues of seven out of eight inoculated calves, with three of these calves also exhibiting pathological changes consistent with chronic paratuberculosis. This indicates that *M.a. paratuberculosis* organisms excreted by infected rabbits have the potential to cause paratuberculosis in cattle. In a parallel experiment, two groups of rabbits were inoculated with either a bovine or leporine-derived isolate of *M.a. paratuberculosis* but no evidence of infection was noted in any animal after a six month incubation period, implying that further, as yet unidentified factors, are involved in the pathogenesis of paratuberculosis in rabbits.

The discovery of paratuberculosis in rabbits has resulted in a reassessment of the natural host range of *M.a. paratuberculosis*. A survey of wildlife in rural Scotland was undertaken to

determine the extent of natural non-ruminant paratuberculosis infection. It revealed evidence of infection in 10 species of wildlife - fox, stoat, weasel, crow, rook, jackdaw, rat, wood mouse, hare and badger. This is the first report of natural *M.a. paratuberculosis* infection in any of these species. The results from this survey were compared with two further investigations. A group of 27 foxes from urban areas of London, with no known exposure to paratuberculosis-affected ruminants or rabbits, were examined, but no evidence of *M.a. paratuberculosis* infection was detected. This implies that *M.a. paratuberculosis* infection of foxes is dependent on contact with infected ruminants or rabbits. However, screening of foxes and rabbits from four paratuberculosis-affected farms in Northern England did not reveal any evidence of natural paratuberculosis, suggesting that the mere presence of paratuberculosis in domestic animals may not be sufficient for infection of wildlife to occur. Further investigations are indicated to clarify the inter-relationships of paratuberculosis cycles in domestic ruminants, rabbits, and other species of wildlife.

Immunological changes following oral inoculation with *M.a. paratuberculosis* were investigated in young lambs. After an incubation period of four weeks, analysis of the lymphocyte subsets present in the jejunal and ileal Peyer's patches and mesenteric lymph node revealed a predominately cell mediated immune response. Using immunohistochemical methods, an increase in the number of $\gamma\delta$ T cells in the jejunal and ileal Peyer's patches was demonstrated, indicating a possible role for this class of T cells in the early immune response to *M.a. paratuberculosis* exposure. No change in the number of $CD1^+$ cells was apparent in the intestinal lymphoid tissue in response to *M.a. paratuberculosis* inoculation.

Preliminary evidence of genetic susceptibility to paratuberculosis was identified in a naturally infected flock of sheep, with the offspring of one ram exhibiting a statistically higher incidence of the disease when compared with the offspring of the other sires ($p=0.019$), indicating that host genotype may have an effect on susceptibility to paratuberculosis. A specific base pair substitution in the natural resistance associated macrophage protein (NRAMP) gene is known to confer susceptibility to murine mycobacterial infection, however, analysis of the genome of 18 paratuberculosis-affected and 60 clinically normal ruminants revealed no evidence of this mutation.

The discovery of infection of non-ruminant wildlife species with *M.a. paratuberculosis*, a significant rise in the numbers of γδ T cells during initial stages of the disease in ruminants, and preliminary evidence indicating that host genotype may have a significant impact on susceptibility to paratuberculosis, has furthered our knowledge of the epidemiology and pathogenesis of this disease, and may aid in the creation of more effective control measures against paratuberculosis.

1 Literature Review

1.1 Paratuberculosis

Paratuberculosis, or Johne's disease, is a chronic enteritis of both wild and domestic ruminants, caused by *Mycobacterium avium* subsp *paratuberculosis* (*M.a. paratuberculosis*). Animals are thought to be infected soon after birth, but clinical disease occurs only after a long incubation period (Figure 1-2). Paratuberculosis was first described in 1895 (Johne and Frothingham 1895) as a derivative of tuberculosis, but by 1914 it had been characterised and the causative organism identified (Twort 1914). However, progress in understanding the epidemiology and pathogenesis of paratuberculosis has been slow and it remains a significant source of economic loss to farms and livestock industries worldwide.

1.2 *Mycobacterium avium* subsp *paratuberculosis*

The *Mycobacterium* genus belongs to the actinomycete group of eubacteria, along with the genera *Nocardia, Rhodococcus* and *Corynebacterium.* All mycobacterium species are aerobic or microaerophilic, non-motile and non-sporing, and possess a highly lipid-rich cell wall which resists staining by the conventional Gram method (Brennan and Nikaido 1995). Mycobacteria are therefore classified as acid-fast bacilli, and routinely stained by the Ziehl-Neelsen (ZN) method. There are currently over 70 recognised species and subspecies of *Mycobacteria* (Inglis 1997), including non-pathogenic saprophytic organisms, opportunistic pathogens, and obligate pathogens. The pathogenic species include *M. tuberculosis* and *M. bovis*, the causative organisms of human and cattle tuberculosis.

Mycobacterium avium subsp *paratuberculosis* is classified as an obligate intracellular pathogen, and has undergone a number of name changes, including *M. johnei* and *M. paratuberculosis.* It is a slow growing species of mycobacterium, taking up to 16 weeks and sometimes longer to grow on specialised culture medium. The bacterium is 0.5-1.5μm in length and requires external sources of both iron and mycobactin for growth. *Mycobacterium avium* subsp *paratuberculosis* is very closely related to two other members of the *M. avium* complex, *M.a. avium* and *M.a.*

sylvaticum, but can be differentiated by a panel of phenotypic properties (Thorel *et al* 1990), or by the presence of the DNA insertion sequence IS900 (Green *et al* 1989).

1.2.1 Survival of the organism in the environment

Mycobacterium avium subsp *paratuberculosis* is resistant to extremes of temperature (Sung and Collins 1998), desiccation, and many chemical disinfectants (Chiodini *et al* 1984). Although accurate measures of the survival of the organism in the environment have not been made, it has been successfully recovered after 17 months in water and 252 days in slurry (Lovell *et al* 1944, Larsen *et al* 1956, Jorgensen 1977). The closely related organism *M.a. avium* is commonly found in water and soil (Collins *et al* 1984, Falkinham, III 1996, Covert *et al* 1999, Sugita *et al* 2000a), underlining the ability of these mycobacterial organisms to persist in the environment. Associations have been identified between the prevalence of paratuberculosis and soil low in calcium, with high acidity, or with increased iron content (Jansen 1948, Kreeger 1991, Johnson-Ifearulundu and Kaneene 1999), suggesting that soil type may influence the survival of *M.a. paratuberculosis* in the environment and hence the prevalence of disease.

1.2.2 Strain typing

Recent studies investigating the strain types of mycobacterial species such as *M. tuberculosis* and *M. bovis* have revealed crucial information about these organisms and the diseases they cause (Krebs 1997, Serraino *et al* 1999). However, *M.a. paratuberculosis* has, comparatively, a much more highly conserved genome which decreases the amount and quality of epidemiological information obtained by typing this organism. Despite this, there have been a number of advances in recent years in the molecular typing of *M.a. paratuberculosis*.

Culturing of *M.a. paratuberculosis* colonies in the laboratory provides evidence of different strains of the organism. Growth times and pigmentation of isolates vary, with some sheep strains exhibiting a yellow colouration and taking up to eight months to grow, compared to four months for the majority of cattle isolates (A. Pirie pers. comm.). Growth requirements for the *M.a. paratuberculosis* strain present in Iceland are so fastidious the organism has not yet been isolated (Fridriksdottir *et al* 1999).

A number of molecular typing techniques have been employed to differentiate between strains, including pulsed field gel electrophoresis (PFGE) (Feizabadi *et al* 1997), restriction fragment

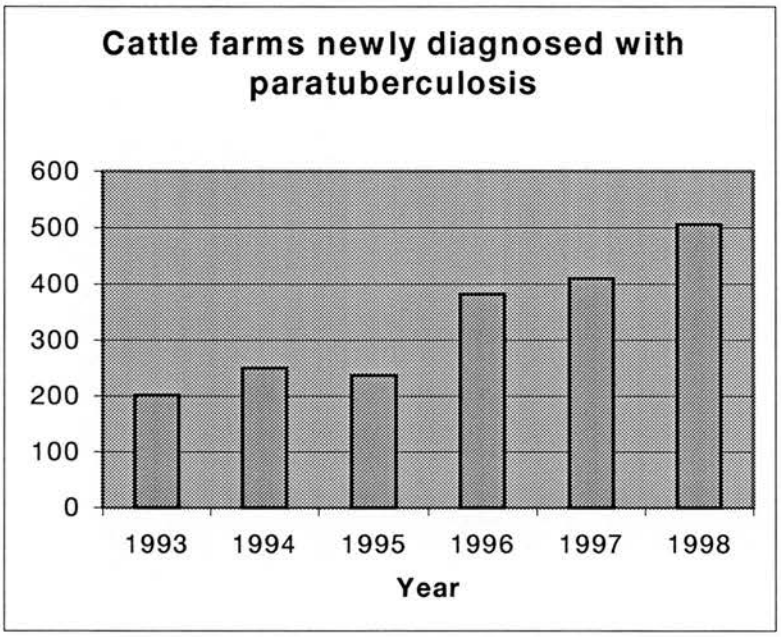
length polymorphism (RFLP) analysis (Collins *et al* 1990, Whittington *et al* 1998a), and chemotyping (Hines and Frazier 1993, Greig *et al* 1999). These techniques have recently been reviewed and compared (Stevenson and Sharp 1997). Some of these strain typing methods utilise the DNA insertion sequence IS900, a 1451 base pair repetitive element found between 14 and 16 times in the genome of the organism (Green *et al* 1989). Six of these insertion sites appear to be variable, allowing for the characterisation of strains of the organism by RFLP analysis incorporating an IS900 probe (Collins *et al* 1990), or a multiplex polymerase chain reaction (PCR) (Bull *et al* 1999). The use of some of these techniques is discussed in section 1.9 below.

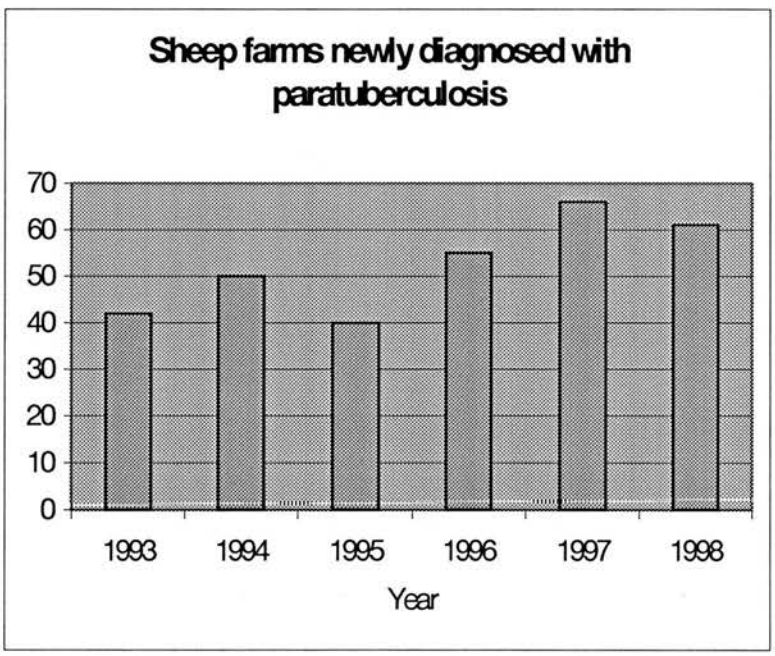
1.3 Prevalence of paratuberculosis

Estimates of the prevalence of paratuberculosis are generally regarded as conservative, since the clinical signs of disease can be subtle and non-specific, and diagnostic tests have low sensitivity. A postal survey of dairy farmers in England and the borders of Wales found that 17.4% had had paratuberculosis in their cattle, with 4.9% reporting at least one clinical case between 1985 and 1994 (Cetinkaya *et al* 1998). This prevalence is lower than the 7.8% reported for cattle in the four years between 1953 and 1957 (Withers 1959). Based on figures published by the Scottish Agricultural College, Veterinary Science Division, the number of cattle and sheep farms newly diagnosed with paratuberculosis has risen steadily between 1993 and 1998 (Anon. 2000) (Figure 1-1).

There is evidence that paratuberculosis is also increasing in prevalence in other countries. In New Zealand a 45% increase in the number of infected sheep flocks and a 66% increase in infected cattle herds was reported between May 1990 and May 1993 (Thompson *et al* 1999). Since the first diagnosis of ovine paratuberculosis in Australia in 1980, infection has spread to 728 flocks in six states (Allworth 2000). The prevalence of paratuberculosis in US dairy herds is reported to be over 20% (Ott *et al* 1999, Wells and Wagner 2000), compared with a reported incidence in 7.9% of beef operations (Dargatz and Wells 1999).

Figure 1-1. The increasing number of farms newly diagnosed with paratuberculosis in Scotland between 1993 and 1998.





1.4 Initial infection

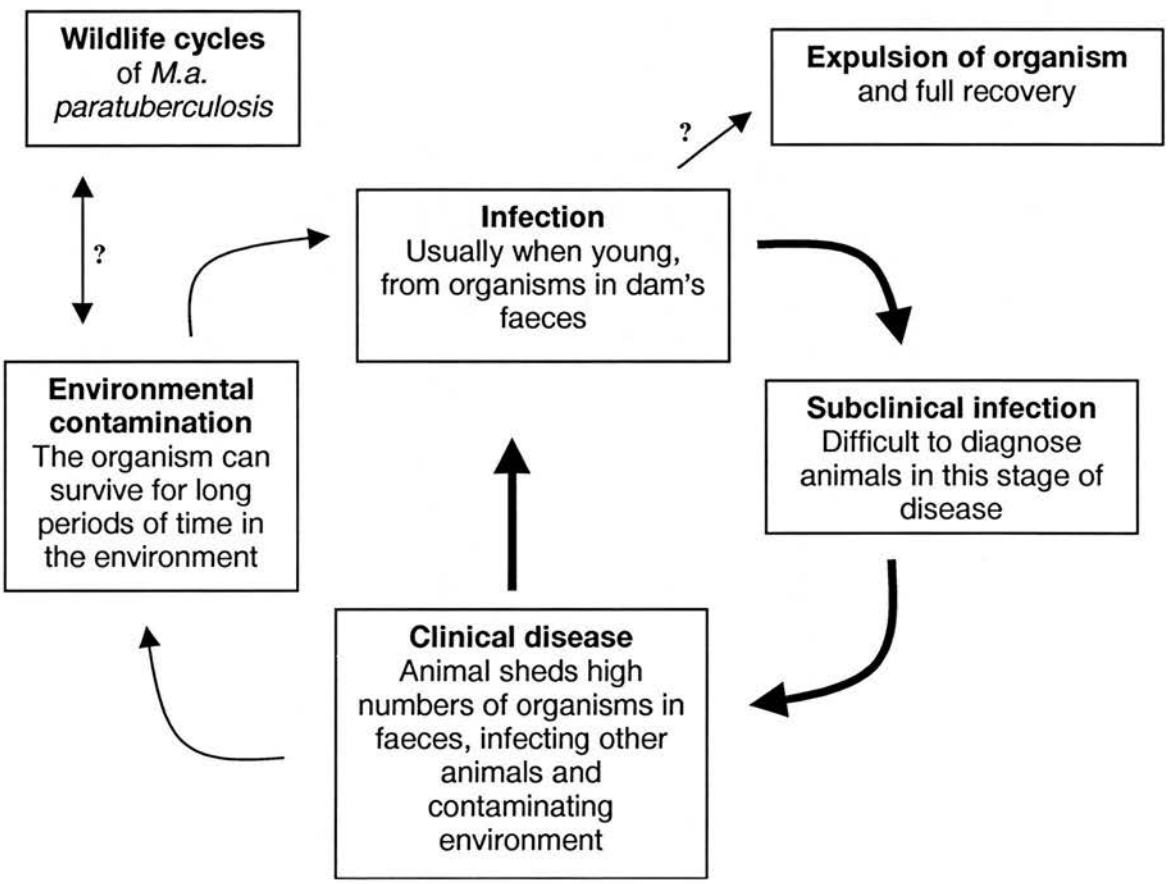
Ingestion of organisms from contaminated faeces is thought to be the most common route of infection, although *M.a. paratuberculosis* has been recovered from the milk (Doyle 1954), semen (Larsen and Kopecky 1970), uterus (Kopecky *et al* 1967) and fetus (Doyle 1958) of infected cattle. Paratuberculosis has been experimentally induced following intratracheal and intravenous routes of inoculation (Kluge *et al* 1968), however, intramammary administration of organisms produced only mild localised lesions (Larsen and Miller 1972), while experimental intrauterine inoculation failed to produce disease in either cow or fetus (Merkal *et al* 1982). In a study of 45 sheep with clinical paratuberculosis, six of which were pregnant, neither gross nor histological lesions were noted in the uterus, fetus or mammary tissue, suggesting that the involvement of these tissues in the transmission of ovine disease is rare (Clarke and Little 1996).

The dose of organisms required to induce infection is unknown and most likely varies, depending on factors such as age, stress, and genetic susceptibility (Clarke 1997). Lambs have been experimentally infected by oral inoculation with as few as 1×10^3 organisms (Brotherston *et al* 1961a). Experimental work has revealed a dose response effect of *M.a. paratuberculosis* inoculation. When lambs were inoculated orally with 10^3, 10^6, or 10^9 organisms, a higher proportion of the lambs given the larger dose became infected, and developed more extensive lesions (Nisbet *et al* 1962). Experiments utilising large doses of inoculum also resulted in the comparatively rapid formation of relatively extensive lesions (Payne and Rankin 1961a, Kluge *et al* 1968, Juste *et al* 1994) (Table 1-1 on page 28).

A number of reviews (Bendixen 1978, Chiodini *et al* 1984, Clarke 1997) have stated that young animals are more susceptible to *M.a. paratuberculosis* infection, and most control programmes are based on this assumption, however, the extent of this susceptibility is unclear. While a number of experiments have indicated that younger animals are more readily infected with *M.a. paratuberculosis* (McEwen 1939, Levi 1948, Taylor 1953a, Rankin 1961, Payne and Rankin 1961a, Payne and Rankin 1961b, Rankin 1962), other studies found no correlation between age at infection and resultant disease (Nisbet *et al* 1962). There are reports of paratuberculosis occuring in animals experimentally inoculated when five months and older, indicating that *M.a. paratuberculosis* is capable of infecting older animals (Doyle 1953, Larsen *et al* 1975, Gilmour

et al 1978). Relevant epidemiological evidence is scant, with only one report describing increased susceptibility of young animals, compared to adults, in a paratuberculosis-affected herd of cattle (Hagan 1938). An investigation into the comparative susceptibility of younger animals to *M.a. paratuberculosis* infection under field conditions is required to clarify this important issue.

Figure 1-2 Hypothetical cycle of paratuberculosis in an infected group of domestic ruminants



Other factors thought to affect the susceptibility of an animal to infection with *M.a. paratuberculosis* include host genotype (chapter 7), intensive farming systems, poor diet (Stabel *et al* 1996a), stress, and concurrent bovine viral diarrhoea virus (BVDV) infection (Clarke 1997, Radostits *et al* 1997). Studies into the early immunopathological changes associated with *M.a. paratuberculosis* exposure and infection (see section 1.7 below) may lead to a clearer understanding of factors affecting the susceptibility of animals to infection with *M.a. paratuberculosis*.

1.5 Clinical signs

Clinical signs of paratuberculosis are usually seen in animals aged two to six years old. The most common signs in cattle are weight loss and diarrhoea, which is usually very fluid ("hosepipe" diarrhoea) with a fetid smell, but contains no discernable blood or mucus (Radostits *et al* 1997). There is no concurrent fever, the appetite and demeanor of the animal are unaffected, and clinical signs are refractory to both antibiotic and anthelminthic treatment. Less common signs include depigmentation and dullness of the coat (Chiodini *et al* 1984). Affected dairy cows exhibit a decrease in milk yield (Benedictus *et al* 1987) as well as a higher incidence of mastitis and infertility (Merkal *et al* 1975).

Sheep also show chronic weight loss (refer to Plate **1,1**) but rarely diarrhoea, although in the terminal stages of disease they may have soft, pasty faeces (Clarke and Little 1996). Other non-specific signs of chronic disease, such as wool slip and submandibular oedema, have been reported (Chiodini *et al* 1984, Clarke 1997, Radostits *et al* 1997, Greig 2000). Goats have been reported to show depression and dyspnoea, as well as chronic weight loss, decreased milk yield and pasty faeces, although diarrhoea is rare (Fodstad and Gunnarsson 1979, Radostits *et al* 1997, Rubin *et al* 1999, Greig 2000).

The clinical signs in both sheep and cattle may be intermittent and appear to be precipitated by increased stress, such as parturition or lactation (Jubb *et al* 1991, Sharp 2000).

Paratuberculosis in deer exhibits some unique features. A more severe form of the disease has been reported in yearlings – these animals lose weight rapidly, and die after only a short period of illness. Older deer exhibit the more chronic syndrome of decreased body weight with retention of the winter coat (Gilmour and Nyange 1989, Reid 1994). Paratuberculosis has been reported in farmed alpacas and llamas, with affected animals showing emaciation and diarrhoea accompanied by a good appetite (Miller *et al* 1999). The thick coat of South American camelids can effectively mask weight loss, rendering detection difficult. Clinical paratuberculosis has been reported in South American camelids as young as 12 months old (Ridge *et al* 1995).

1.6 Pathology

1.6.1 Gross Pathology

The gross pathology of paratuberculosis in all species is that of a chronic enteritis. Lesions are most common in the distal ileum and ileocaecal valve, with a thickened, velvety and hyperaemic mucosa thrown into transverse corrugations (Buergelt *et al* 1978, Clarke and Little 1996). Similar lesions, which can be continuous or segmental, may also be found in other regions of the intestine, from the duodenum to the rectum, although their severity has been correlated with their distance from the terminal ileum. Infection with a pigmented strain of *M.a. paratuberculosis* results in discolouration of the mucosa, ranging from light yellow to a deep orange, most commonly seen in ovine paratuberculosis (Clarke and Little 1996) (Plate **1,2**). The mesenteric and sometimes ileocaecal lymph nodes are enlarged and oedematous, and the lymphatics draining the intestine can be dilated and inflamed (Plate **1,3**). There may be atrophy of fat and skeletal muscle, increased serosal fluid in the abdominal cavity, and evidence of scouring on the perineum of cattle.

The gross pathology found in paratuberculosis bears no correlation to the severity of either the clinical signs or histopathological changes present (Hallman and Witter 1933, Carrigan and Seaman 1990, Clarke and Little 1996, Clarke 1997) - a severely wasted sheep with pasty faeces and marked anorexia may have only subtle gross lesions on post mortem examination. Similarly, an animal with mild or undetectable gross changes may have histological evidence of extensive chronic inflammation in the intestines and associated lymph tissue.

Comparatively, the macroscopic lesions of paratuberculosis in sheep and goats have been judged more subtle than in cattle (Little 1997, Sharp 2000), and the gross changes accompanying cases of ovine multibacillary disease more extensive and easily detectable than those of the paucibacillary form (Clarke and Little 1996).

1.6.2 Ovine histopathological changes

A number of classification systems have been suggested to cover the range of histopathological changes seen in sheep with paratuberculosis, some of which encompass only clinically affected animals, while others include subclinical forms (Stamp and Watt 1954, Rajya and Singh 1961, Reddy *et al* 1984, Carrigan and Seaman 1990, Clarke and Little 1996, Perez *et al* 1996). Subclinically affected animals usually show no gross lesions, and the histopathology is either mild or moderate. Mild lesions consist of a small number of macrophages forming small granulomata in, or associated with, the lymphoid tissue of the intestine (Perez *et al* 1996). Moderate lesions have numerous large granulomata extending into the adjacent mucosa and the draining lymph nodes, usually with small numbers of acid fast bacteria (AFB) visible (Rajya and Singh 1961).

Marked histopathological changes have been categorised as either multibacillary or paucibacillary. The multibacillary (also known as lepromatous) form is the most common, occuring in approximately 70% of cases (Clarke and Little 1996). The lamina propria of the intestine is closely packed with macrophages containing a clear, large nucleus, and abundant, slightly foamy cytoplasm which stains lightly with haematoxylin and eosin (H&E). The arrangement of these cells, also known as epithelioid cells, produces a mosaic-like appearance to the mucosa (Plates **1,4** and **1,6**). A small number of lymphocytes, neutrophils, eosinophils and plasma cells have also been reported in the inflammatory infiltrate. The villi of the intestines are stunted and fused, with flattened, wide apices. The few crypts present can be either compressed, distended with cellular debris, or abscessated. Clusters of epithelioid cells extend through the submucosa and into the serosa. Serosal oedema, dilation of lymphatic vessels, and an increase in the number of lymphocytes, especially around the blood and lymphatic vessels, are present. Focal aggregates of epithelioid cells in the walls of the lymphatic vessels, and the presence of lymphatic thrombi, consisting mainly of macrophages, have been noted (Carrigan and Seaman 1990, Perez *et al* 1996). Giant cells are rarely seen in cases of multibacillary paratuberculosis. Large numbers of AFB are present in the epithelioid cells in the mucosa (Plates **1,5** and **1,7**),

although fewer are visible in the submucosa and serosa. The draining lymph nodes exhibit generalised lymphocytic hyperplasia, with large macrophages, usually aggregated in granulomata of variable size, found throughout the cortex. Smaller numbers of these cells are noted in the subcapsular and medullary sinuses. Fewer AFB are present in the lesions of the lymph nodes when compared to the intestinal lesions. The serosa of the lymph nodes exhibits similar lesions to the serosa of the intestines.

The paucibacillary, or tuberculoid, form of the disease occurs in 30% of cases (Clarke and Little 1996). The intestinal villi are stunted and fused, with small lymphocytes the predominant inflammatory cell present (Plates **1,8** and **1,10**). A variable number of macrophages are also present, either individually or arranged in small, well-delineated but non-encapsulated granulomata. The lesions extend into the submucosa, with a variable number of lymphocytes and plasma cells present. The lesions in the serosal layer are similar to those described in the multibacillary form of the disease. The associated lymph nodes exhibit multifocal granulomata, most commonly in the paracortical and interfollicular areas. The presence of numerous large Langhans giant cells, some with more than 30 nuclei, have been reported in the lymph nodes of some paucibacillary cases (Perez *et al* 1996). Far fewer AFB are present in the intestines and lymph nodes of paucibacillary cases, with none identified in some cases (Plates **1,9** and **1,11**).

There are intermediate histopathological forms of ovine paratuberculosis, sharing features of both multibacillary and paucibacillary forms. Only one type of pathology is seen in the intestines of each animal, but an affected flock may contain animals with either form of the disease. A similar spectrum of histopathological changes are seen in human leprosy, caused by *M. leprae* (Ridley 1974).

1.6.3 Bovine histopathological changes

There have been fewer reports of investigations into the histopathological changes occurring in cattle with natural paratuberculosis. One study examined 32 cattle, including cases of both clinical and subclinical paratuberculosis, and classified the changes into three groups, mild, moderate and marked (Buergelt *et al* 1978). The most commonly affected part of the intestines in all three forms was the distal small intestine, with no changes identified in the duodenum and, in most cases, lesions stopping abruptly at the level of the ileocaecal valve (ICV). The histopathological changes were described as follows.

i. Mild lesions were found only in the villous lamina propria, or paracortical areas of lymph nodes, and consisted of an occasional Langhans giant cell or epithelioid cell, with very few AFB present.
ii. Moderate lesions extended through the lamina propria to the submucosa, and consisted of groups of epithelioid and Langhans giant cells. These lesions were also found in the subcapsular sinus, as well as the paracortical areas of the lymph node and the liver. There were a greater number of AFB present when compared to the mild lesions.
iii. Marked lesions consisted of many macrophages and giant cells, spread throughout the four layers of the intestines - the lamina propria, submucosa, tunica muscularis, and serosa. The villi were stunted, crypt glands distended, and the lumen of lymphatic vessels swollen. The Peyer's patches were surrounded by inflammatory cells and there were numerous AFB visible. No lesions corresponding to the "tubercular" form of leprosy were noted in any of the 32 animals examined and no caseous necrosis, mineralisation, or ulceration was present. Other reports differ from the above description, with some workers detailing necrotic lesions (Hallman and Witter 1933), extensive changes in the upper portion of the large intestine (Barker *et al* 1993), and the presence of paucibacillary forms of bovine paratuberculosis (Kreeger 1991).

The presence of mineralisation and necrosis has been cited as a distinguishing factor between ovine and bovine paratuberculosis (Jubb *et al* 1991). However, while foci of caseous necrosis, sometimes with mineralisation, have been noted in both natural and experimental cases of ovine paratuberculosis (Stamp and Watt 1954, Nisbet *et al* 1962), as well as in affected goats (Fodstad and Gunnarsson 1979, Little 1997, Corpa *et al* 2000), these changes have been absent in other studies (Clarke and Little 1996, Perez *et al* 1996) or attributed to other causes, such as intestinal parasitism (Carrigan and Seaman 1990).

1.6.4 Caprine histopathological changes

In a recent report examining 68 goats from paratuberculosis-affected flocks (Corpa *et al* 2000), histological lesions were similar to those of sheep, with multibacillary, paucibacillary and an intermediate form described. Necrosis, with and without calcification, was frequently seen in the intestines and associated lymph nodes of the goats, a feature which has been reported in previous caprine studies (Fodstad and Gunnarsson 1979, Little 1997). Some unique changes have been reported in cases of caprine paratuberculosis, including axonal degeneration of sciatic and

brachial plexus nerves, and amyloidosis in the kidneys, adrenal and mammary glands (Chiodini *et al* 1984).

1.6.5 Cervine histopathological changes

The histopathology of paratuberculosis in deer is comprised of accumulations of epithelioid cells, macrophages and giant cells in the intestines and associated lymph nodes. The number of AFB present can be variable (Reid 1994). The histopathological lesions of *M.a. paratuberculosis* infection in deer are similar to those caused by *M.a. avium* infection, consequently, culture of tissues is recommended to enable distinction between these two diseases.

1.6.6 Histopathological changes in South American camelids

The histopathology in alpacas and llamas has not been reported in detail, but there is no evidence for any unique features in these species (Miller *et al* 1999).

1.6.7 Lesions in other tissues

There have been numerous reports of paratuberculosis-like pathology affecting organs other than the intestines and associated lymph nodes. Natural paratuberculosis of the liver, reported in sheep (Reddy *et al* 1984, Clarke and Little 1996), cattle (Taylor 1953a, Buergelt *et al* 1978), goats (Little 1997) and llamas (Ridge *et al* 1995), consists of small clusters of macrophages scattered throughout the parenchyma, with low numbers of intracellular AFB present. Systemic paratuberculosis, associated with a bacteraemia and widespread lesions, is thought to occur in end stage disease. *Mycobacterium avium* subsp *paratuberculosis* has been demonstrated in the bloodstream of clinically affected cattle (Koenig *et al* 1993) and lesions from organs including the lung and draining lymph nodes, spleen and kidneys have been reported in severe cases of ovine and bovine paratuberculosis (Reddy *et al* 1984, Hines *et al* 1987, Carrigan and Seaman 1990, Ridge *et al* 1995, Clarke and Little 1996, Whitlock *et al* 1996). There is also a report of hepatic encephalopathy associated with paratuberculosis in a goat (Rubin *et al* 1999).

1.7 Immunopathogenesis of paratuberculosis

After initial infection, paratuberculosis, like most mycobacterial diseases, is characterised by a long subclinical period which can last for years, followed by chronic, progressive clinical disease. Crucial features of the pathogenesis of this disease which have yet to be elucidated include the immunological changes that occur during initial infection, the mechanisms that allow the bacterium to resist destruction by the host macrophages, and the trigger(s) that result in the onset of clinical disease.

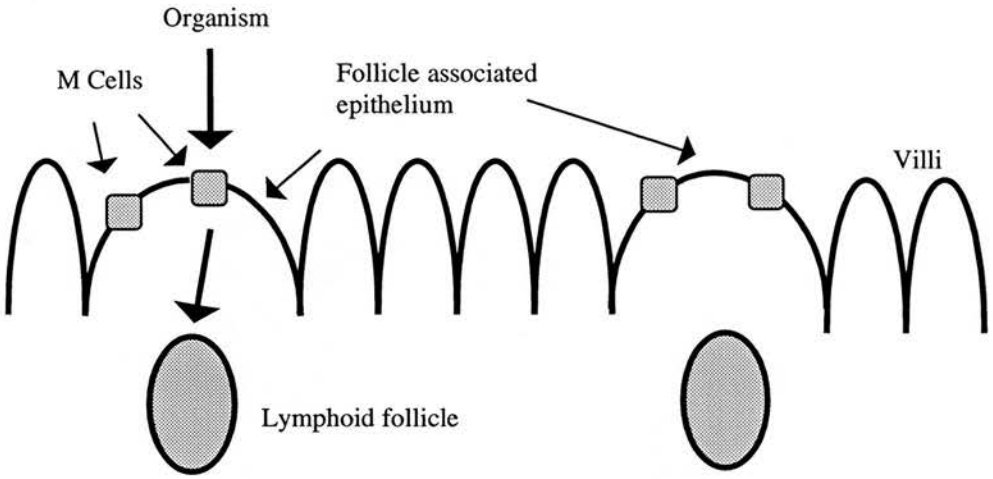
1.7.1 Early *M. a. paratuberculosis* infection

While recent work has focused on events occurring in end stage, clinical paratuberculosis (Little *et al* 1996, Clarke and Little 1996, Burrells *et al* 1999), few studies have investigated earlier changes. It is believed that initial exposure to *M.a. paratuberculosis* results in a course similar to that described in other mycobacterial diseases, such as tuberculosis in humans and badgers (Gallagher *et al* 1998), where infection can be followed either by expulsion of the organism and full recovery, or long term subclinical infection (Figure 1-2). This hypothesis therefore renders the events occuring during the initial stage of infection important in determining the outcome of infection. However, little is known about the factors influencing the animal's ability to repel, or alternatively to succumb to the initial *M.a. paratuberculosis* challenge.

1.7.1.1 Peyer's patches

Peyer's patches are organised groups of lymphoid follicles found in the small intestine (Plates **6,1** and **6,2**). They initially increase in number after birth, before declining as maturity is reached (Fujimura and Owen 1996). In most species, Peyer's patches contain B cells which proliferate when stimulated with foreign antigens. However, the ruminant ileal Peyer's patch (IPP) is a primary source of B lymphocytes, analogous to the Bursa of Fabricius in birds (Reynolds and Morris 1983). While the jejunal Peyer's patch (JPP) of ruminants functions as in other species, remaining present and active throughout the life of the animal, the IPP regresses as the animal reaches sexual maturity so that by 18 months, there is little follicular structure remaining (Reynaud *et al* 1991).

Figure 1-3 Possible route of entry of *M.a. paratuberculosis* from the gut lumen, across the follicle associated epithelium via the M cells, and into the underlying lymphoid follicles that make up the Peyer's patches of the intestines.



The epithelium overlying the lymphoid follicles of the JPP and IPP is known as the follicle associated epithelium (FAE). Scattered amongst the epithelial cells in the FAE are M cells (Figure 1-3), which act as portals of entry across the mucosa, taking up various molecules from the intestinal lumen, and transporting them to the underlying lymphoid tissue. This "antigen sampling" role may assist the development of a competent and well developed local immune system. However, a number of pathogens have been shown to breach the gut mucosa through M cell endocytosis, including *M. tuberculosis* (Teitelbaum *et al* 1999), *Brucella abortus* (Ackermann *et al* 1988), and others (Fujimura and Owen 1996, Lugton 1999). *Mycobacterium avium* subsp *paratuberculosis* is also thought to cross the intestinal mucosa via M cells, with electron microscopy studies revealing intact *M.a. paratuberculosis* as well as vacuoles containing debris possibly of bacterial origin in the cytoplasm of M cells (Momotani *et al* 1988) (Figure 1-3).

1.7.1.2 Pathology of early paratuberculosis

There have been numerous experiments charting the changes occurring in animals after inoculation with *M.a. paratuberculosis*. However, inoculation routes, regimes, species and ages of animals and monitoring techniques varied widely between the studies, consequently, care must be taken when interpreting and comparing the results (Table 1-1).

The earliest published lesions found in reponse to *M.a. paratuberculosis* inoculation were 16 and 18 days post infection (pi) (Kluge *et al* 1968, Begara-McGorum *et al* 1998), and were comprised of small granulomata, consisting mainly of macrophages with abundant pink cytoplasm, as well as a small number of polymorphonuclear cells, in the interfollicular and follicular areas of the JPP, IPP and mesenteric lymph node (MLN). Studies have also reported the gradual expansion of lesions to the areas adjacent to the Peyer's patches, and eventually areas of intestine distant from the lymphoid tissue (Juste *et al* 1994, Perez *et al* 1996). Some workers noted isolated granulomata in the ICV region as early as 45 days pi (Juste *et al* 1994), in the lamina propria of the jejunum 17 weeks pi (Sigurdardottir *et al* 1999) and the tips of the intestinal villi at eight months pi (Angus and Gilmour 1971). Very few if any AFB are reported by workers examining these early lesions. Gross lesions appear later than histopathological changes, with thickening of the intestinal wall and accompanying corrugation of the mucosa reported at six (Nisbet *et al* 1962) and 13 months pi (Gilmour *et al* 1978).

There have been reports of isolation of *M.a. paratuberculosis* from a variety of organs following experimental infection, including the retropharangeal lymph node (Brotherston *et al* 1961b, Juste *et al* 1994, Sigurdardottir *et al* 1999) and tonsils (Levi 1948). The significance of such findings is unclear.

Table 1-1 Summary of published oral experimental infections with *M.a. paratuberculosis*

species	infective dose used	age at infection	infection regime	animals first examined	lesions first noted	reference
lambs	from $1x10^3$ org	variable	variable	1m	1m	(Brotherston *et al* 1961a)
lambs	$1x10^8$ org	10wo	one dose	1m	1m	(Brotherston *et al* 1961b)
lambs	variable	7d-3mo	variable	1w	4m	(Nisbet *et al* 1962)
lambs	200mg org	3wo	one dose	1d	16d	(Kluge *et al* 1968)
lambs	$1x10^9$org	8wo	once a week for 10w	8m	8m	(Gilmour and Angus 1971, Angus and Gilmour 1971)
lambs	$1x10^9$ org in total	5mo	once a week for 10w	5m	5m	(Gilmour *et al* 1978)
lambs	150mg wet weight or $1.36x10^6$cfu	4mo	daily for 2d	15d	45d	(Juste *et al* 1994)
lambs	$1x10^9$cfu	5do	on d0, 2 and 4	3d	18d	(Begara-McGorum *et al* 1998)
calves	100mg organism	0-6mo	one dose	24m	>2yr	(Taylor 1953a)
calves	200mg wet weight	<3mo	one dose	1d	1m	(Payne and Rankin 1961a)
calves	group1: 2g wet weight group 2: 20g infected mucosa	2mo	weekly for 3w	21m	21m	(McDonald *et al* 1999)
goats	24-43mg moist growth	1-18mo	3-8 doses spread over 1-2w	unclear	unclear	(Levi 1948)
goats	10mg dry weight	7-25do	every day for 10d	3w	4m	(Sigurdardottir *et al* 1999)
deer	$1x10^8$cfu	2wo	once a week for 10w	10m	10m	(Burrells *et al* 1996)

Abbreviations used:

d: days w: weeks m: months yr: years cfu: colony forming unit
do: days old wo: weeks old mo: months old org: organisms

There was often disparity between histopathological lesions and corresponding positive cultures in the experiments. One study (Begara-McGorum *et al* 1998) reported lesions in the IPP of four out of eight infected animals, however, no positive cultures were obtained from any of these areas. The same study did not find any pathological changes indicative of *M.a. paratuberculosis* infection in the MLN of any of eight infected animals, but cultured *M.a. paratuberculosis* from the MLN of three. Similar inconsistencies have been reported between culture and histopathological results following inoculation of young goats with *M.a. paratuberculosis* (Sigurdardottir *et al* 1999).

1.7.1.3 Host immune response to early infection

The early immune response to mycobacterial infection has been shown to be predominately cell mediated (Dannenberg, Jr. 1991, Orme 1993, Cooper and Flynn 1995, Schaible *et al* 1999). The importance of the initial cell mediated immune (cmi) response has been used to help develop a hypothesis for the lesion distribution in early paratuberculosis. It has been suggested that the lower percentage of T cells found in the ruminant IPP, when compared with the JPP, may result in a reduction in cmi, and therefore create a more favourable environment for persistence of *M.a. paratuberculosis* (Miyasaka *et al* 1983). Local immune tolerance has also been presented as a possible explanation for the "favoured site status" of the IPP. The involution of the IPP at approximately six months of age has been linked to the relative resistance of older animals to infection with *M.a. paratuberculosis* and cited as further evidence of the importance of IPP in the early stages of *M.a. paratuberculosis* infection (Nisbet *et al* 1962). However, these theories do not explain why early lesions of paratuberculosis are found in the JPP and MLN as well as the IPP.

It is possible that initial infection with *M.a. paratuberculosis* triggers specific local changes in the JPP, IPP and MLN which are critical in determining the subsequent pattern of disease development. A recent study (Begara-McGorum *et al* 1998) examined both systemic and intestinal immunological reactions to *M.a. paratuberculosis* infection in neonatal lambs eight weeks after experimental infection. Infected lambs had higher numbers of $CD8^+$ and $CD2^+$ lymphocytes in the JPP, and fewer B cells in the IPP and MLN when compared with control lambs. This resulted in an increase in the T/B cell ratio in the gut associated lymphoid tissue (GALT), further indicating that the early response to *M.a. paratuberculosis* infection is predominantly cell mediated. Significantly, this study found that, in contrast to the local

changes, no alteration in the peripheral blood lymphocyte (PBL) numbers occurred, highlighting the autonomy of the GALT, and the importance of investigating local gut immunity rather than relying on predictions extrapolated from systemic changes.

The study also examined cytokine changes in early *M.a. paratuberculosis* infection by comparing the amount of mRNA in the GALT of infected and uninfected lambs. This revealed an increase in the cytokines granulocyte macrophage colony stimulating factor (GM-CSF) and tumour necrosis factor alpha (TNF-α), and a decrease in interleukin-4 (IL-4) and gamma interferon (IFN-γ). These changes can be analysed with reference to the Th0, Th1 and Th2 categorisation of immune responses. $CD4^+$ lymphocytes of mice have been classified as either Th1 or Th2, depending on their pattern of cytokine production (Mosmann *et al* 1986). Th1 $CD4^+$ lymphocytes produce cytokines such as IFN-γ, IL-2 and TNF-α, and are associated with macrophage activation and cmi, while Th2 $CD4^+$ lymphocytes produce cytokines including IL-4, IL-5, IL-10 and IL-13, and are associated with antibody production, allergic reactions, and defence against helminth parasites (Abbas *et al* 1994). Th0 lymphocytes produce detectable levels of both Th1 and Th2 cytokines. Th1 cytokines inhibit the proliferation and cytokine secretion of Th2 cells and vice versa (Pearlman *et al* 1993). The cytokine changes described in the study above (Begara-McGorum *et al* 1998) contain features of both a Th1 response (increased TNF-α and decreased IL-4) and Th2 response (decreased amounts of IFN-γ), and therefore suggest that the early immunological changes in response to *M.a. paratuberculosis* infection involve complex cytokine interactions which cannot be classified in a simple fashion.

1.7.1.4 Interactions between macrophages and M.a. paratuberculosis.

Electron microscopy studies have identified *M.a. paratuberculosis* in intracellular compartments of macrophages in the Peyer's patch of the gut soon after experimental infection (Bendixen *et al* 1981, Momotani *et al* 1988), however, the techniques and processes involved in the paradoxical intracellular survival of mycobacteria in inflammatory cells is poorly understood. The receptors involved in the entry of the organism into the macrophage are unknown, but may involve cell-associated receptors, such as CD14 (Wright 1995) and macrophage scavenger receptors (Schaible *et al* 1999) or cell free serum components, including immunoglobulins or complement receptors. The addition of serum to *in vitro* models of *M.a. paratuberculosis*-macrophage infection has been found to enhance the early uptake of the organism into the cell (Zurbrick and

Czuprynski 1987), supporting claims that these opsonizing receptors are involved in the uptake of the bacteria into host cells.

The techniques that enable the organisms to resist the killing mechanisms of the cell are similarly poorly understood, and may include prevention of phagosome-lysosome fusion, production of glycolipid, or alteration of the environment of the phagosome (Russell 1998, Schaible *et al* 1999). It has even been suggested that the mere physical presence of the organism in the phagosome can interrupt maturation of the organelle (de Chastellier *et al* 1995).

1.7.2 Subclinical infection

During the long incubation period, which represents the second phase of paratuberculosis, the host immune response and mycobacteria appear to be in equilibrium, with the persistence of organisms in a small, focal area of inflammation (Perez *et al* 1996). One study examined a group of subclinically affected sheep, and reported a higher level of cmi, as determined by measurement of delayed-type skin hypersensitivity, in animals with fewer organisms present (Gilmour *et al* 1978). This early work was later supported by studies reporting higher levels of IFN-γ, and therefore cmi, in subclinically affected cattle when compared to both uninfected and clinically affected animals (Sweeney *et al* 1998, Koets *et al* 1999, Stabel 2000) suggesting that adequate cmi allows the animal to keep *M.a. paratuberculosis* in check during the long incubation period.

The trigger which produces the shift from subclinical to clinical disease is, as yet, unknown. *In vitro* studies have shown that $CD4^+$ T cells from paratuberculosis-affected animals produce more IFN-γ than either $CD8^+$ or γδ T cells (Bassey and Collins 1997), however, decreased numbers of $CD4^+$ lymphocytes are noted in clinical multibacillary disease (Little *et al* 1996, Navarro *et al* 1998). The reduction in the number of $CD4^+$ lymphocytes in diseased animals may result in a decrease in IFN-γ, producing a waning of cmi, thus allowing *M.a. paratuberculosis* to multiply, leading to clinical disease. However, it is not known why the $CD4^+$ cells decrease. It has been hypothesised that, rather than a decrease in the number, a change in the type of $CD4^+$ lymphocytes is the critical factor controlling the shift from subclinical to clinical disease (Clarke 1997), and that the observed decrease in IFN-γ indicates a shift from a Th1 to a Th2 type of immune response. Mice and humans with latent tuberculosis have been shown to produce

predominately Th1 cytokines, while a Th2 profile is evident during clinical disease (Torres *et al* 1998, Howard and Zwilling 1998, Howard and Zwilling 1999). It is possible that a similar situation exists in paratuberculosis, with a Th1 to Th2 shift marking the onset of clinical multibacillary disease.

Other processes have been implicated in the reduction of cmi and onset of clinical disease, including a decrease in the immunomodulatory effect of $CD8^+$ lymphocytes on the activity of γδ T cells (Chiodini and Davis 1992, Chiodini and Davis 1993), or a decrease in the responsiveness of B cells to *M.a. paratuberculosis* antigen (Waters *et al* 1999). The events responsible for the change from subclinical infection to paucibacillary disease are poorly understood.

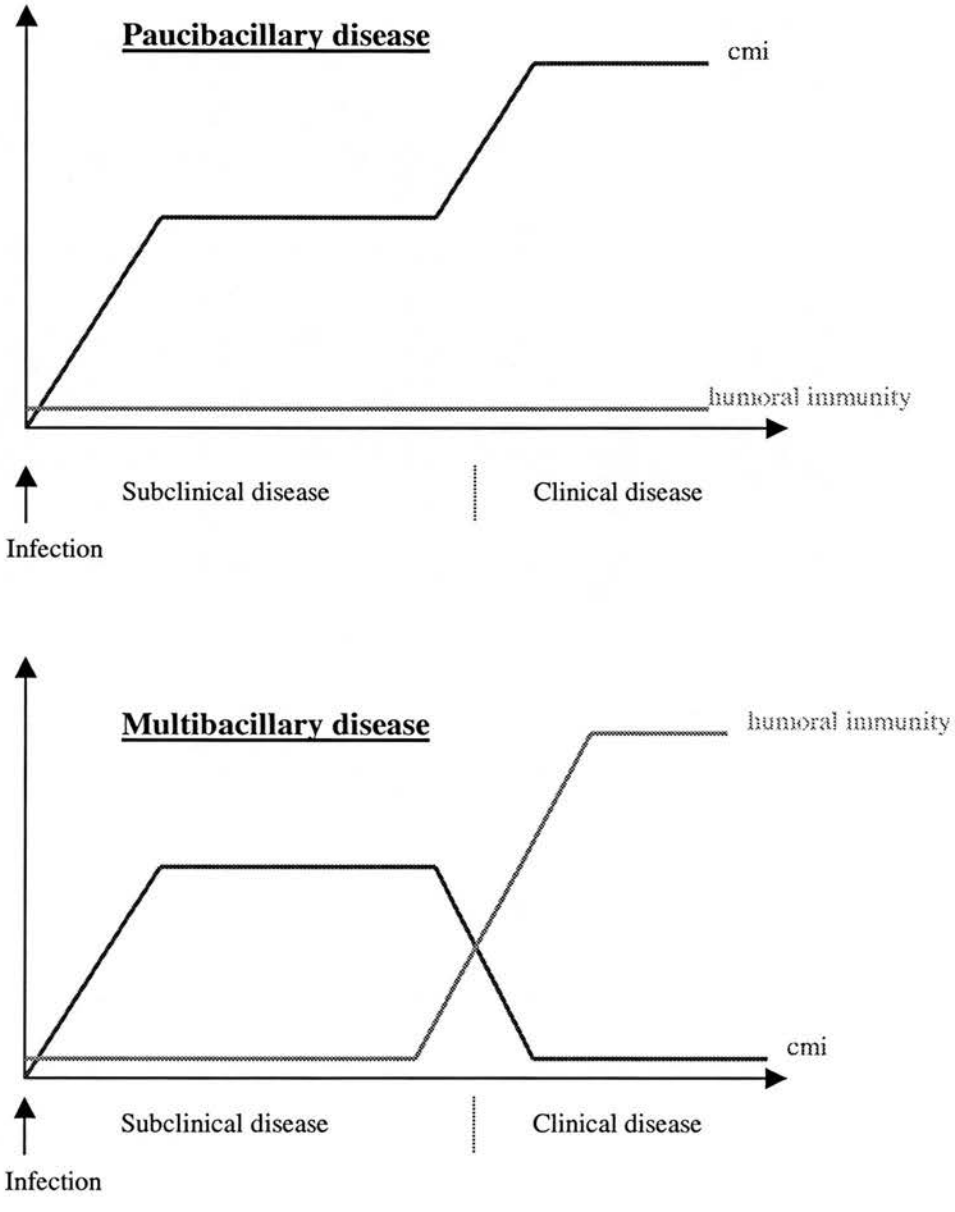
1.7.3 Clinical disease

A spectrum of histopathological changes have been reported in both leprosy and paratuberculosis, the two ends of which have been designated paucibacillary and multibacillary (see section 1.6.2 above). Various studies on the immune status of patients and animals with either form have resulted in the hypothesis that they are the result of opposite immunological excesses (Figure 1-4).

At the onset of multibacillary paratuberculosis, a shift from a cell mediated to a humoral immune response is thought to occur. However, the humoral immune response is unable to control the disease, and the animal succumbs to an overwhelming multiplication of the organism (Clarke 1997). In the less common paucibacillary form the immunopathology is less well understood. It has been suggested that, while the multibacillary form is due to an excessive humoral immune response, the paucibacillary form is the result of an excessive cmi response (Clarke and Little 1996).

This theory has been supported by a number of recent reports including investigations into the response of lymphocytes from paratuberculosis-affected sheep to Johnin pure protein derivative (J-PPD), and the determination of the levels of IFN-γ in paratuberculosis-affected sheep. Both techniques revealed a higher level of cmi in sheep with paucibacillary lesions when compared to sheep with the multibacillary form (Burrells *et al* 1998, Perez *et al* 1999).

Figure 1-4 A diagrammatic comparison of cell mediated and humoral immune responses in sheep developing either paucibacillary or multibacillary paratuberculosis



Th1 and Th2 cytokine profiles have been linked with the two forms of histopathology noted in leprosy, as patients with paucibacillary leprosy lesions have been reported to exhibit increased levels of the Th1 cytokines IL-2 and IFN-γ, while patients with multibacillary lesions show higher levels of the Th2 cytokines IL-4, IL-5, and IL-10 (Yamamura *et al* 1991, Schlienger *et al* 1998).

Studies investigating the cytokine profiles present in the two forms of paratuberculosis have found some results analogous to those reported in leprosy, with investigations into *in vitro* levels of cytokine production from lymphocytes revealing that sheep with paucibacillary lesions produce higher levels of the Th1 type cytokines IFN-γ and IL-2 when compared to either control or multibacillary-affected sheep (Burrells *et al* 1999). However, while providing clear evidence of the role of Th1 cytokines in paucibacillary paratuberculosis, this investigation was unable to determine the level of Th2 cytokines in multibacillary disease. Only two reports have directly investigated the level of Th2 cytokines in paratuberculosis-affected animals (Alzuherri *et al* 1996, Begara-McGorum *et al* 1998). The latter restricted the analysis to changes occurring during the first eight weeks pi (see section 1.7.1.3 above), and the former examined levels of only one Th2 cytokine – transforming growth factor beta (TGF-β), as well as three Th1 cytokines. Using semi-quantitative analysis of the amount of mRNA present, these workers recorded the levels of Th1 cytokines TNFα, IL-1β and IL-6, as well as the Th2 cytokine TGF-β, in sheep with multibacillary or paucibacillary paratuberculosis. They found that animals with paucibacillary infections had significantly higher levels of TNF-α compared to control animals, but did not show raised levels of IL-1β, while sheep with multibacillary infections showed a significant rise in the levels of all three Th1 cytokines, but no increase in the level of TGF-β. These results do not appear to support the hypothesis that multibacillary disease is due to a high Th2 response, and paucibacillary disease to a high Th1 response.

There is also conflicting evidence regarding the role of Th1 and Th2 cytokines in human tuberculosis. Studies report increased levels of both Th1 and Th2 cytokines in peripheral blood mononuclear cells (PBMC) of patients with clinical tuberculosis (Barnes *et al* 1993a, vanCrevel *et al* 2000, Yamada *et al* 2000, Seah *et al* 2000), as well as at the site of infection (Orme *et al* 1993, Barnes *et al* 1993b, Fenhalls *et al* 2000). While the conflicting results may be a reflection of confounding factors such as the nutritional status of the patient, concurrent parasitic infection,

or the response to therapy (Barnes and Wizel 2000), they highlight the potential difficulties when trying to simplify intricate immunopathological changes. While it is tempting to subscribe to the "Th1 good, Th2 bad" mnemonic, the reality is probably much more complex (Allen and Maizel 1997).

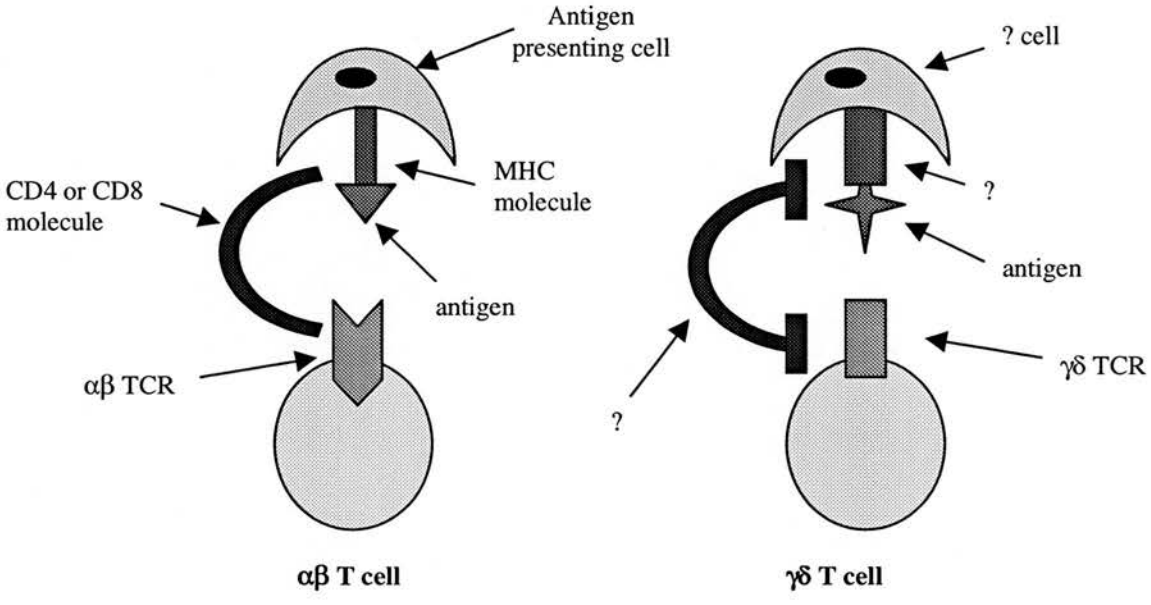
While further research into paratuberculosis increases our knowledge of the pathogenesis of the disease, it is still not known if the multibacillary and paucibacillary forms of paratuberculosis are sequential or divergent. The presence of intermediate forms of pathology suggest that transition between the two types can occur, with one study reporting that sera from a minority of sheep with tubercular lesions showed a strong response in the agar gel immunodiffusion (AGID) test (Clarke and Little 1996). The authors suggested that this was due to antibodies left over from a previous lepromatous response. It should be noted that this finding is in contrast to other studies (Perez *et al* 1996). The reasons behind the development of these two different histopathological forms, with indistinguishable clinical disease but apparently polarised immunological activity, is one of the most intriguing conundrums of this disease, and one which deserves significant research effort.

1.7.4 CD1 molecules and γδ T cells

There is increasing evidence of involvement of both CD1 molecules and γδ T cells in the immune response to mycobacterial infections. Gamma delta T cells have been shown to increase in number in response to mycobacterial infection (Janis *et al* 1989), and CD1 molecules have the ability to present mycobacterial cell wall components to cells of the host's immune system (Sieling *et al* 2000).

Gamma delta T cells were first reported in 1986 (Brenner *et al* 1986). Rather than expressing the more common αβ T cell receptor (TCR), they express a γδ TCR. Unlike the αβ T cell, which uses a CD4 or CD8 molecule to interact with the major histocompatibility complex (MHC) molecule on the APC, allowing recognition of the antigen, and subsequent clonal expansion of the T cell, the vast majority of γδ T cells do not express either a CD4 or CD8 molecule ("double negative"), and thus are MHC class unrestricted. The APCs and antigens involved in γδ T cell recognition and responses are yet to be fully elucidated, hampering our understanding of the exact function of these cells (Figure 1-5).

Figure 1-5. A comparison of the components involved in the interaction of an antigen with either an αβ or a γδ T cell



The CD1 molecule acts as a third class of antigen presenting molecule (APM), analogous to MHC class I and II, has a similar structure to the MHC molecule (Zeng *et al* 1997), and is expressed on the surface of "professional" APCs (Porcelli 1995). However, unlike MHC molecules, some CD1 molecules have been shown to present lipid instead of protein antigens to T cells, thereby expanding the range of antigens the immune system can recognise and respond to (Sugita *et al* 2000b).

1.7.4.1 Function of γδ T cells

The major function of γδ T cells is thought to be the early defence of mucosal surfaces against infectious agents (Hein and Mackay 1991). The cells are found in greatest proportion at mucosal surfaces including the skin and gut, and have been shown to proliferate in response to various infectious agents (Table 1-2), including a number of intracellular bacteria. The rapid rise in γδ T cells seen in response to infectious diseases has led some workers to suggest that they bridge a gap in the immune response between the early non-specific reponse, and the later αβ T cell response.

Table 1-2 Organisms that have been found to cause an *in vivo* increase in γδ T cells

Organism	Reference
M. tuberculosis	(Janis *et al* 1989)
M. bovis BCG	(Inoue *et al* 1991, Hoft *et al* 1998)
M. bovis	(Pollock *et al* 1996, Cross *et al* 1996, Smith *et al* 1999)
M. leprae	(Modlin *et al* 1989, Sieling *et al* 1999)
Leishmania donovani	(Raziuddin *et al* 1992)
Salmonella choleraesuis	(Emoto *et al* 1992)
Brucella melitensis	(Bertotto *et al* 1993)
Listeria monocytogenes	(Munk *et al* 1996, JouenBeades *et al* 1997)
Coxiella burnetii	(Schneider *et al* 1997)
Francisella tularensis	(Poquet *et al* 1998)
Plasmodium falciparum	(Ho *et al* 1994)
Sendai virus	(Ogasawara *et al* 1994)

Gamma delta T cells have been shown to exhibit cytolytic activity against a variety of targets, including *M. tuberculosis*-infected macrophages (Tsukaguchi *et al* 1995, Lang *et al* 1995, Kabelitz *et al* 1999), and have the ability to secrete both Th1 and Th2 cytokines (Kaufmann 1996), implying that they have a direct effect on cells harbouring intracellular pathogens, as well as an indirect role, modulating the hosts immune defences via production of inflammatory mediators. Recent work has even suggested that their primary role may be as either stimulators (Soltys and Quinn 1999) or inhibitors (McCole *et al* 1999) of other immune responses. Although γδ T cells increase in number in an early response to infection, they do not appear to play a crucial role in "memory" immune responses. A rapid but transient increase in the number of γδ T cells in the intestinal villi of calves experimentally infected with *Cryptosporidium parvum* has been shown, however there was no corresponding increase when the animals were re-infected (Abrahamsen *et al* 1997, Abrahamsen 1998).

A variety of molecules have been shown to stimulate γδ T cells, including proteins, lipids (Tanaka *et al* 1995), and even autologous molecules (Havran *et al* 1991). Changes in γδ T cells have been detected in a variety of diseases including cancer, auto immune diseases (Born *et al* 1990, Born and Obrien 1990, Haregewoin *et al* 1991), and stress (Rajasekar *et al* 1990, Baldwin *et al* 2000). It is clear from the varied roles and stimuli associated with them that γδ T cells are

likely to participate in a number of immune functions, and further investigation of this enigmatic T cell subset will help elucidate their role.

1.7.4.2 Ruminant γδ T cells

Most of our knowledge of γδ T cells has arisen from human or murine studies, however, care must be taken when extrapolating from these fields, since significant interspecies differences exist:

→Gamma delta T cells are more populous in ruminants, constituting 30% of the total circulating lymphocyte population in 3-4 month old lambs, compared to 1-5% in humans and mice (Hein and Mackay 1991).

→Unlike human and murine γδ T cells, ruminant γδ T cells do not express CD2 or CD6 molecules, although the majority do express a unique surface molecule WC1 (sometimes referred to as T19 in sheep) (Wijngaard *et al* 1992, Machugh *et al* 1997).

→Ruminant γδ T cells have been found to express exceptionally diverse antigen receptors, indicating a greater recognitive capacity than other species (Evans *et al* 1994, Hein and Dudler 1997).

→Ruminant γδ T cells are not thought to contribute significantly to IFN-γ production, unlike the situation in humans and mice (Bassey and Collins 1997).

These differences, depicting a group of populous and variable γδ T cells in ruminant species, suggest that these cells may play a more important role than in other species.

Investigations into γδ T cells in ruminants are summarised in Table 1-3. Gamma delta T cells have been found to increase in these animals in response to a range of infectious agents, including *M.a. paratuberculosis* and *M. bovis*. Investigation of cattle experimentally infected with *M. bovis* found the levels of γδ T cells in peripheral blood initially decreased and then increased, suggesting localisation to developing lesions, followed by clonal expansion (Pollock *et al* 1996). No significant differences were found in the numbers of circulating γδ T cells in a similar experiment involving deer (Cross *et al* 1996), however it was suggested that because eight weeks had elapsed between infection and sampling, this study missed the changes noted by Pollock and workers.

Table 1-3. Summary of studies on ruminant γδ T cells

Disease / organism	Outcome	Reference
M.a. paratuberculosis	γδ T cells may play a cytotoxic, immunoregulatory role in late paratuberculosis	(Chiodini and Davis 1992, Chiodini and Davis 1993)
M.a. paratuberculosis	increase in γδ T cells in both forms of the disease	(Little *et al* 1996)
M. bovis	initial decrease, then increase in γδ T cells in peripheral blood in early infection	(Pollock *et al* 1996)
Theileria parva	γδ T cells are involved in immune response	(Daubenberger *et al* 1999)
Mannheimia haemolytica A1	γδ T cells increase in BAL[1] fluid	(McBride *et al* 1999)
Mastitis	increase in γδ in peripheral blood and milk	(Soltys and Quinn 1999)
Enzootic Bovine Leukosis	decreased γδ T cells in late neoplastic lesions	(Chiba *et al* 1995)
Caprine Arthritis and Encephalitis Virus (CAEV)	increased γδ T cells in goats with chronic CAEV infection	(Jolly *et al* 1997)
BVDV superinfection	increase in γδ T cells in some persistently infected animals	(Bruschke *et al* 1998)
Bovine Respiratory Syncytial Virus	no change in γδ T cell numbers early in infection	(Mcinnes *et al* 1999)
Eimeria ovinoidalis	general increase in gut γδ T cells	(Aleksandersen *et al* 1995)
Trichostrongylus colubriformis	γδ T cell depletion reduced faecal egg count	(McClure *et al* 1996)
Ostertagi ostertagii	increase in γδ T cells in response to infection	(Almeria *et al* 1997)
Cryptosporidium parvum	rapid, transient increase in intestinal γδ T cell numbers	(Abrahamsen *et al* 1997, Abrahamsen 1998)
Fasciola hepatica	may limit host damage	(McCole *et al* 1999)
Stress / environmental change	decrease in γδ T cells	(Baldwin *et al* 2000)
Delayed Type Hypersensitivity (DTH)	no evidence for γδ T cell role in ovine DTH	(Pyrah and Watt 1995)

[1]BAL: bronchoalveolar lavage

1.7.4.3 Mycobacterial disease and γδ T cells

In 1989, the first studies describing a link between γδ T cell numbers and mycobacterial infection were published. An increase was reported in the number of γδ T cells in the draining

lymph nodes of mice immunised with *M. tuberculosis*, and also in the lungs of mice exposed to aerosolised *M. tuberculosis* (Janis *et al* 1989, Augustin *et al* 1989). Since then, studies have shown that γδ T cells respond both *in vivo* and *in vitro* to mycobacterial stimulation (Table 1-2)(Boom 1999). Direct evidence of γδ T cell action against mycobacteria was recently reported, when it was shown that they lyse *M. tuberculosis*-infected macrophages via the granule exocytosis pathway, and also release effector molecules such as perforin, to kill the *M. tuberculosis* organisms (Dieli *et al* 2000).

Recent work has suggested that γδ T cells, while playing an as yet unspecified role in early mycobacterial infection, may also be important in the later stages of disease. Phenotypically active γδ T lymphocytes were reported in the peripheral blood of patients with tuberculosis (BehrPerst *et al* 1999), suggesting a persistent role for γδ T cells throughout clinical tuberculosis, and not just during the early, acute phase.

1.7.4.4 Mycobacterium avium *subsp* paratuberculosis *infection and γδ T cells*

Gamma delta T cell numbers have been reported to increase in the JPP, IPP and MLN of lambs in the eight weeks following experimental infection with *M.a. paratuberculosis*, although these increases did not reach statistical significance (Begara-McGorum *et al* 1998). Other investigations into γδ T cells in paratuberculosis have concentrated on the role of these cells in chronic, endstage disease. Gamma delta T cells from chronically infected animals have been found to perform an immunoregulatory function *in vitro*, with cytotoxic effects towards $CD4^+$ cells subject to downregulation by $CD8^+$ cells (Chiodini and Davis 1992, Chiodini and Davis 1993). Investigations into the number and distribution of lymphocyte subsets in the ileum of paratuberculosis-affected sheep found an increase in the density of γδ T cells in paucibacillary lesions, and a relative increase in the percentage of γδ T cells in the multibacillary form of the disease (Little *et al* 1996), possibly indicating that γδ T cells play a role in the immunopathology of endstage paratuberculosis. A recent study inoculated γδ knockout (KO) mice with *M.a. paratuberculosis* (Tanaka *et al* 2000), and reported that granulomatous lesions in the liver of the KO mice were fewer and smaller when compared to changes in the wildtype mice. The authors suggested that γδ T cells may therefore play a crucial role in the formation of the epithelioid granulomata in both early and later stages of ruminant paratuberculosis.

1.7.4.5 CD1 molecules and mycobacterial infection

The CD1 family can be divided into two groups - group 1 (CD1a, CD1b and CD1c) and group 2 (CD1d). Group 1 CD1 molecules are found in a number of species including man and ruminants but are not retained in rodents. Group 2 CD1 molecules in contrast have been found in all species in which the CD1 family has been investigated to date (Porcelli 1995). A RNA transcript exists for a fifth type - CD1e, but no protein has yet been identified (Calabi *et al* 1989). Sheep possess at least seven CD1 genes, including homologues of CD1B and CD1D (Rhind *et al* 1996, Rhind *et al* 1999, Hopkins *et al* 2000).

The first indication of the role CD1 might play in the immune response to mycobacterial disease was the demonstration of a human T cell clone that recognised mycobacterial antigens in a CD1 restricted manner (Porcelli *et al* 1992). CD1 has since been found to present, to cells of the immune system, a number of non-peptide lipids found in the mycobacterial cell wall, including lipoarabinomannan, mycolic acids, and phosphatidylinositol mannosides (Beckman *et al* 1994, Sieling *et al* 1995, Beckman *et al* 1996, Sugita *et al* 1998).

Modulation of CD1 expression has been described in association with several mycobacterial infections. In leprosy there is upregulation of group 1 CD1 molecules in the paucibacillary form of the disease which is characterised by a strong cmi response, but in the multibacillary form of the disease, characterised by low levels of cmi and rapid multiplication of the *M. leprae* organism, no corresponding increase in the number of $CD1^+$ cells was detected (Sieling *et al* 1999). Paratuberculosis has a similar spectrum of lesions to leprosy and preliminary studies have indicated upregulation of CD1 expression in both tuberculoid and lepromatous paratuberculosis (S. Rhind, pers. comm.)

Three recent reports have provided further evidence linking CD1 molecules and immunity to mycobacterial infections. Using mass spectrometry, one study revealed that CD1c proteins recognise isoprenoid glycolipids, a class of mycobacterial phospholipid antigens, and that the response of PBLs from *M. tuberculosis*-sensitised patients to these glycolipids is significantly greater than the response of PBL isolated from *M. tuberculosis*-naive patients (Moody *et al* 2000). An anti-CD1c monoclonal antibody was found to block these responses, illustrating for the first time CD1-mediated T cell activity in response to mycobacterial disease in humans. In a

second study, three cell lines of $CD4^+$ lymphocytes isolated from leprosy lesions were shown to recognise mycobacterial antigen in a CD1-restricted fashion (Sieling *et al* 2000). Two cell lines were CD1b restricted, and one CD1c restricted, but all three responded to mycobacterial antigens by producing a Th1 pattern of cytokines, suggesting that these CD1-restricted $CD4^+$ lymphocytes play a role in immunity against mycobacterial infection. Finally, in a simpler study, it was found that mice showed increased susceptibility to *M. tuberculosis* infection when treated with anti-CD1 antibodies (Szalay *et al* 1999).

The complexity of the CD1-mycobacteria relationship is hinted at in a report by Stenger and co-workers. Infection of APCs with *M. tuberculosis in vitro* was shown to downregulate the expression of human CD1b, suggesting an immune evasion mechanism of *M. tuberculosis*, and thereby implying that CD1 molecules are detrimental to *M. tuberculosis* (Stenger *et al* 1998). However, this view is challenged by a recent review which suggests that *M. tuberculosis* utilises CD1 molecules to its advantage, activating them via lipid antigens present in the mycobacterial cell wall, and thus encouraging an active immune response, granuloma formation, and development of a necrotic lesion, providing the organism with a suitable environment for extended periods of survival (Shinkai and Locksley 2000).

1.7.4.6 CD1 molecules and γδ T cells

There is growing evidence that γδ T cells and CD1 molecules are linked in the immune responses to mycobacterial infection. Numerous CD1-restricted γδ T cell lines have been identified (Porcelli *et al* 1989, Faure *et al* 1990, Sugita *et al* 1998), and a recent report described a group of γδ T cells with the ability to recognise CD1 molecules without the presence of a foreign antigen (Spada *et al* 2000). This suggests that either an endogenous lipid antigen, or no antigen at all in the binding groove of CD1 molecules is capable of stimulating γδ T cells. This study also reported that the activated γδ T cells released granulysin, a known antimycobacterial agent. The authors conjecture that mycobacterial infection may result in increased expression of CD1 molecules which are capable, even without a mycobacterial lipid antigen in their binding groove, of activating γδ T cells, encouraging them to release anti-bacterial cytokines and inflammatory mediators, providing a very rapid response to infection. However, it is still not clear whether this response assists the host in killing the mycobacteria, or whether in fact it aids the survival of the organism by creating a suitable environment for intracellular survival

(Shinkai and Locksley 2000). If this interaction occurs during early *M.a. paratuberculosis* infection, it may explain why so few intracellular AFB are noted in the well formed granulomata in the gut lymphoid tissue (Begara-McGorum *et al* 1998).

It is clear that both γδ T cells and CD1 molecules play a role in the immune response to mycobacterial infection, however, the exact nature of these roles, whether they are linked, or even if they assist the host or pathogen, is not known.

1.8 Diagnosis

One of the major problems underlying any control plan or epidemiological study of paratuberculosis is the lack of an antemortem diagnostic test with high specificity and sensitivity. Subclinically infected animals and clinical cases with paucibacillary lesions are especially difficult to detect. The most suitable test for the diagnosis of paratuberculosis varies with each situation, depending on how many animals are to be tested, their value, and the expertise available.

The methods available include serology, serum biochemistry, detection of cmi, PCR, culture and histopathology.

1.8.1 Serology.

These tests indicate the level of antibody to *M.a. paratuberculosis* antigens. As no *M.a. paratuberculosis*-specific antigen is known, all available tests suffer from a lack of specificity. They also may produce false negatives in subclinical and paucibacillary cases. Anergic animals with end-stage clinical disease will also have low to undetectable levels of *M.a. paratuberculosis* antibodies. Available tests include the complement fixation test, enzyme-linked immunosorbent assay (ELISA), and AGID. These assays utilise a number of antigens with a wide range of different test protocols. These have been compared by a number of workers under varying circumstances (Milner *et al* 1989, Shulaw *et al* 1993, Clarke *et al* 1996, Thoen and Haagsma 1996, Perez *et al* 1997). Some tests are available commercially, and present a relatively cheap and simple diagnostic method (Ellis *et al* 1998), most useful when trying to estimate the prevalence of paratuberculosis in a herd or flock.

1.8.2 Serum biochemistry

Clinically affected sheep have been shown to have decreased serum calcium levels, markedly decreased albumin, but normal globulin levels (Jones and Kay 1996). Therefore a simple laboratory test, highlighting this difference between the albumin and globulin levels, has the potential to differentiate between paratuberculosis and other causes of wasting, such as parasitic gastroenteritis.

1.8.3 Detection of cmi

These tests have been proposed as a solution to the problem of low sensitivity of serological tests in early *M.a. paratuberculosis* infection, when there are low antibody titres, but detectable cmi. The tests for cmi include intradermal skin tests, lymphocyte stimulation assays (McDonald *et al* 1999), IFN-γ assays (Burrells *et al* 1995, Stabel 1996, Sweeney *et al* 1998) and IL-2R expression (Whist *et al* 2000). However, the intradermal skin test has very low specificity and interferes with the tuberculin test (Kreeger 1991, Hietala 1992, Perez *et al* 1999), while the other tests require further optimisation before routine use is practical.

1.8.4 Polymerase chain reaction (PCR)

The most specific test for *M.a. paratuberculosis* is detection by PCR of the IS900 insertion sequence (Green *et al* 1989, Collins *et al* 1990, Stevenson and Sharp 1997). It has the advantage that it can be performed on bacterial cultures, tissues (Cetinkaya *et al* 1996, Gwozdz *et al* 1997), milk (Grant *et al* 1998), blood, faeces or formalin fixed, paraffin wax embedded tissues (Plante *et al* 1996, Miller *et al* 1997, Whittington *et al* 1999a). Mycobacterial DNA from ZN smears has even been successfully multiplied using the PCR technique (Rossi *et al* 2000). However, PCR is expensive, requires to be undertaken by a skilled technician (Collins 1996) and has low sensitivity, although extraction of the organisms may be enhanced by immunomagnetic separation (Grant *et al* 1998). The sensitivity of the PCR used with tissues or faecal samples may be decreased by the presence of inhibitors such as bile salts or large amounts of irrelevant DNA. Recent work suggests other mycobacterial species have IS900-like sequences, which may produce false positive results (Cousins *et al* 1999). Restriction of the PCR product, careful selection of primers or use of ELISA-based detection systems should overcome this problem.

1.8.5 Culture

Culture of *M.a. paratuberculosis* is labour-intensive, time-consuming and expensive, involving a long decontamination procedure (Whipple *et al* 1991). Ovine strains appear to be more fastidious in their nature than bovine strains of *M.a. paratuberculosis* (Carrigan and Seaman 1990, Shulaw *et al* 1993, Choy *et al* 1998), and thus non-specialised culturing of ovine faeces is not recommended as a diagnostic tool. Culturing of cattle faeces is more sensitive but still can take up to 20 weeks. Recently the "Bactec" culturing technique, based on detection of radiolabelled CO_2 produced in response to mycobacterial growth in radiometric liquid media, has improved the sensitivity, especially in the case of ovine isolates (Whittington *et al* 1998b, Whittington *et al* 1999b). These systems have the added advantage of providing much quicker results when compared with conventional culture techniques. Intermittent shedding of organisms can lower the sensitivity of all culture techniques (Chaitaweesub *et al* 1999, McDonald *et al* 1999), however, recent studies suggest pooling faecal samples in both the radiometric systems and traditional culture methods may decrease costs and labour without sacrificing sensitivity (Kalis *et al* 1999, Whittington *et al* 2000a).

1.8.6 Histopathology

In many cases this is the most certain method of diagnosing paratuberculosis, as the organism shows a high propensity for the area involving the ICV and associated lymph nodes, where it produces pathognomonic lesions (see section 1.6 above). However, both gross and histopathological changes can be subtle in subclinically affected animals (Perez *et al* 1996, McDonald *et al* 1999). The ZN stain is used to identify AFB in lesions, although immunohistochemistry (Stabel *et al* 1996b) or *in situ* PCR techniques (Sanna *et al* 2000) have been advocated to improve the sensitivity and specificity of detection of organisms (Plante *et al* 1996, Coetsier *et al* 1998, Brees *et al* 2000). Faecal smears and gut impression smears stained by the ZN method can give a rapid answer and require less expertise than histopathological examination although they are much less sensitive, especially in animals shedding low numbers of organisms.

1.9 Interspecies transmission of *M.a. paratuberculosis*

One of the most widely debated topics concerning the epidemiology of paratuberculosis is the role of interspecies transmission of *M.a. paratuberculosis*. There is anecdotal evidence of *M.a. paratuberculosis* passing between a number of species, including cattle and sheep (Ris *et al* 1987), cattle and goats (Ris *et al* 1988), cattle and deer (Chiodini and Vankruiningen 1983), deer and rabbits (Angus 1990), cattle and rabbits (Greig *et al* 1997) and sheep and llamas (Miller *et al* 1999). The history of paratuberculosis in Iceland provides further evidence of interspecies transmission. The disease on the island is believed to be the result of one point-source of infection associated with the importation of 20 Karakul sheep in 1933. The single strain of organism introduced has not yet been successfully isolated, and shows a greater pathogenicity in sheep, although disease in cattle, goat, and possibly reindeer, has been reported (Gunnarsson 1979, Fridriksdottir *et al* 1999).

Strain typing of *M.a. paratuberculosis* isolates in Australia have shown that "cattle" and "sheep" isolates can be differentiated using restriction enzyme analysis of IS1311 (Whittington *et al* 1998a). This suggests that these two species are infected by distinct strains of the organism and that interspecies transmission is rare, as there are only a small number of cases of cattle strains occurring in sheep, or sheep strains occurring in cattle (Whittington 2000, Whittington *et al* 2000b). In a survey of rabbit and cattle strains from Tayside in Scotland (Greig *et al* 1999), PFGE, RFLP and chemotyping did not identify any species-specific strains of *M.a. paratuberculosis*. In a separate study using RFLP typing, it was found that isolates from cattle and sheep from the same farm displayed the same pattern, possibly indicating interspecies transmission (Pavlik *et al* 1995). A larger survey, analysing RFLP patterns from a number of farms in Argentina, also found the same pattern in both cattle and deer (Moreira *et al* 1999). The authors suggest that this indicates that the cattle could be the source of infection for the deer. However, the inability of strain typing methods to distinguish isolates of *M.a. paratuberculosis* from different species does not prove that interspecies transmission occurs, or give any indication regarding its frequency. It is possible that, as discussed in section 1.2.2 above, currently available typing methods are not sufficiently discriminatory to identify species specific strains of *M.a. paratuberculosis*.

Experimental work has been used to investigate the pathogenicity of *M.a. paratuberculosis* strains in different species. The pigmented sheep strain in the United Kingdom (UK) has produced lesions in an experimentally infected cow (Taylor 1953b), cattle have developed paratuberculosis after inoculation with intestinal material from sheep with clinical paratuberculosis (McEwen 1939), and both sheep and rabbits have developed disease following oral inoculation with a bovine strain of *M.a. paratuberculosis* (Kluge *et al* 1968, Mokresh *et al* 1989, Mokresh and Butler 1990). While these experiments have shown that it is possible for a strain of the organism to cause disease in more than one species, they are based on artificial dose rates and regimes, and so may not accurately reflect situations that occur in the field.

1.10 Wildlife Reservoirs of Paratuberculosis

The epidemiology of paratuberculosis is not fully understood, including the presence and importance of wildlife reservoirs of infection. Control and eradication plans have been proposed which rely on the absence of a significant sylvatic cycle of *M.a. paratuberculosis,* however, no large scale investigation into paratuberculosis in wildlife has been reported. *M.a. paratuberculosis* infection has been found in a number of free-living ruminant populations in a variety of ecological habitats, and recent investigations have revealed natural *M.a. paratuberculosis* infection in wild rabbits in Scotland (Angus 1990, Greig *et al* 1997, Greig *et al* 1999), suggesting that non-ruminant, as well as ruminant wildlife, could play a role in the epidemiology of this disease (Figure 1-2, page 18).

1.10.1 Mycobacterial infections of carnivores

Wildlife reservoirs are suspected to play a critical role in bovine tuberculosis. *Mycobacterium bovis* is enzootic in free-living badgers (*Meles meles*) in areas of the UK (Wilesmith 1991), and circumstantial evidence of infection being passed from badgers to cattle exists (Krebs 1997, Hutchings and Harris 1999). Similarly, brush-tailed possums *(Trichosurus vulpecula*) in New Zealand are considered to be a major wildlife reservoir of *M. bovis* infection (Caley 1998, Kao and Roberts 1999). *Mycobacterium bovis* infection has also been reported in coyotes (*Canis latrans*) in the United States (BruningFann *et al* 1998), a stoat (*Mustela erminea*) and free-living ferrets (*Mustela furo*) in New Zealand (Cooke *et al* 1993), a lynx (*Lynx lynx*) in Spain (Briones *et al* 2000) as well as the domestic cat (Monies *et al* 2000), the mole (*Talpa europaea*), fox (*Vulpes vulpes*), mink (*Mustela vison*), rat (*Rattus norvegicus*), and ferret (*Mustela putorius*

furo) in the UK (Krebs 1997). There are therefore numerous incidents of carnivores being naturally infected with *M. bovis*, although infection is usually at a low prevalence. However, the situation is not entirely clear, as demonstrated in a large survey of wild mammals in the early 1980s in East Sussex. Tissues from nine foxes, 103 rats, 13 weasels, and one stoat were cultured, and no mycobacterial isolates were grown (Wilesmith *et al* 1986). In another survey, no positive cultures were grown from the tissues of five foxes and two badgers (BruningFann *et al* 1998). The particular circumstances rendering carnivores susceptible to *M. bovis* infection are therefore still unclear.

1.10.2 Mycobacterium avium subsp. *paratuberculosis* in free-living populations

1.10.2.1 Ruminant wildlife

Paratuberculosis in ruminant wildlife is well documented, with evidence of infection reported in a range of free-living populations (Table 1-4).

Table 1-4 Free-living ruminants that have been diagnosed with *M.a. paratuberculosis*.

Animal		**Reference**
Bison	*Bison bison*	(Buergelt and Ginn 1999, Ellingson *et al* 1999)
Buffalo (water)	*Bubalus bubalus*	(Lillini *et al* 1999)
Deer (axis)	*Axis axis*	(Riemann *et al* 1979)
Deer (fallow)	*Dama dama*	(Riemann *et al* 1979)
Deer (roe)	*Capreolus capreolus*	(Sharp *et al* 1997, Anon. 1999)
Deer (red)	*Cervus elaphus*	(Sharp *et al* 1997, Anon. 1999)
Deer (sika)	*Cervus nippon*	(Sharp *et al* 1997)
Deer (tule elk)	*Cervus nannodes*	(Jessup *et al* 1981)
Deer (white tailed)	*Odocoileus virginianus*	(Chiodini and Vankruiningen 1983)
Goat (Rocky Mountain)	*Oreamnos americanus*	(Williams *et al* 1979)
Ibex	*Capra ibex*	(Anon. 1999)
Reindeer	*Rangifer tarandus*	(Gunnarsson 1979)
Sheep (big horn)	*Ovis canadensis*	(Williams *et al* 1979)
Moufflon	*Ovis musimon*	(Pavlik *et al* 1999)

1.10.2.2 Marsupials

Mycobacterium avium subsp. *paratuberculosis* has been isolated from the intestinal tissues of a Tammar wallaby (*Macropus eugenii*) and a faecal sample from an eastern grey kangaroo (*Macropus giganteus*). Both animals were collected from properties in Australia with a history of ovine paratuberculosis. In both cases no histological lesions suggestive of paratuberculosis

were noted in the tissues of the animals and it was concluded that the organisms were merely passing through the intestinal tract, rather than being part of an active infection (K. Abbott pers. comm.).

1.10.3 Mycobacterial infections of lagomorphs

There are two reports of *M. bovis* infection of free-living rabbits and hares in New Zealand (Anon. 1980), as well as studies of experimental infection of rabbits with *M. bovis* by aerosolisation resulting in the development of pulmonary cavitary tuberculosis, demonstrating the susceptibility of rabbits to the organism (Cooke *et al* 1993, Converse *et al* 1996, Converse *et al* 1998). In a survey of 285 hares *(Lepus europaeus)* from southern England, mycobacterial species were cultured from five animals - four of the isolates were identified as *M avium* type 2, but the fifth was not classified (Matthews and Sargent 1977). This latter strain showed features consistent with *M.a. paratuberculosis*, such as slow growth and mycobactin dependence, but failed to produce clinical signs of paratuberculosis in inoculated cattle over a 34 month period. In another study tissues from 32 wild rabbits were cultured, but no mycobacteria colonies were grown (Wilesmith *et al* 1986).

1.10.3.1 Mycobacterium avium *subsp.* paratuberculosis *infection of wild rabbits*

In 1990, a report was published describing a case of suspected paratuberculosis in a rabbit from a property in Scotland which had recently experienced an outbreak of paratuberculosis in farmed deer (Angus 1990). The acid fast organisms seen in histological sections were not cultured, but widespread infiltration of the lamina propria of the intestine with large pale staining epithelioid macrophages containing large numbers of AFB was consistent with lesions seen in ruminant paratuberculosis. This report instigated two surveys of free-living rabbits in Scotland. In the first, rabbits were examined from four farms in the Tayside area of Scotland, three with a history of paratuberculosis and one without. Paratuberculosis was diagnosed by both culture and histopathology in rabbits from all four farms. The overall prevalence of paratuberculosis in the 33 rabbits collected was 67% (Greig *et al* 1997). The second, larger survey examined 210 rabbits from 22 farms in seven regions of Scotland - Orkney, the Highlands, Grampian, Dumfries and Galloway, Tayside, Lothian, and the Borders. *Mycobacterium avium* subsp. *paratuberculosis* was cultured from rabbits in the Tayside area, and a further isolate was recovered from a rabbit in the Borders area of Scotland. A mycobacterial species was cultured from a rabbit in Orkney, but due to desiccation of the medium, it could not be typed. Tayside was found to be a "hotspot"

for paratuberculosis infection of rabbits, with *M.a. paratuberculosis* cultured from 53% of rabbits. Analysis of the results from this survey showed that rabbits were significantly more likely to be infected with *M.a. paratuberculosis* if they were collected from a farm which had a history of bovine paratuberculosis. Molecular typing of the isolates from rabbits, cattle and sheep from the farms involved in the survey did not reveal consistent evidence of species-specific strains of *M.a. paratuberculosis*. This statistical and molecular evidence provided support for the hypothesis that rabbits transmit *M.a. paratuberculosis* to the domestic ruminant population.

1.11 Experimental paratuberculosis in laboratory animals

Experimental work with *M.a. paratuberculosis* has revealed that non-ruminant species, including mice, hamsters, guinea pigs, rats and rabbits, can become infected (Chiodini *et al* 1986). Isolation of *M.a. paratuberculosis* and associated histopathological lesions have also been described after experimental infection of lemmings (*Dicrostonyx rubricatus*) (Larsen and Miller 1979). An outbreak of *M.a. paratuberculosis* infection in a laboratory colony of stumptailed macaques (*Macaca arctoides*) has been reported, but the source of infection was never identified (McClure *et al* 1987). More recent work involving laboratory animals has concentrated on the responses of in-bred and genetically modified mice, such as the C57BL/6 strain (Veazey *et al* 1995a) and gene knockout mice (Tanaka *et al* 2000), to *M.a. paratuberculosis* inoculation.

Experimental infection of rabbits with *M.a. paratuberculosis* has been successfully achieved by a number of workers. While some researchers have used intraperitoneal and intravenous inoculation techniques (Twort 1914, Francis 1943, Rankin 1958) this review will concentrate only on those in which oral inoculation was used. Slight lesions were induced in three out of seven rabbits (Harding 1959) and in "several" out of 22 rabbits and seven out of 15 hamsters, following challenge of the young animals with between six and nine doses of *M.a. paratuberculosis* (Hirch 1956). Paratuberculosis was produced in 62% of newborn rabbits (13 out of a total of 21 inoculated) dosed orally with either five or 10 daily doses of 7.8×10^7cfu ml^{-1} of *M.a. paratuberculosis*, starting on the day of birth (Mokresh *et al* 1989), and in five out of sixteen kits challenged with approximately $3x10^8$cfu ml^{-1} on days one and two after birth (Mokresh and Butler 1990). The lesions in the infected rabbits, after an incubation period of 11 or 12 months, consisted of well demarcated granulomata, consisting mainly of macrophages with

an occasional giant cell, in the sacculus rotundus and appendix. Sparse to moderate numbers of intracellular AFB were present in the lesions. The bacteria recovered were very small (1mm diameter) and required up to 15 months growth before becoming visible. They were classified as *M.a. paratuberculosis* on the basis of "characteristic colonies". A further report of experimental paratuberculosis in rabbits described clinical signs of disease, and histopathological changes after an incubation period of between 42 and 70 days following oral challenge with a caprine isolate of *M.a. paratuberculosis* (Mondal and Sinha 1992). Under certain conditions, therefore, rabbits are susceptible to infection with *M.a. paratuberculosis*.

1.12 Genetic resistance

1.12.1 Genetic resistance to infectious disease in humans

Genetic resistance to infectious diseases is a recognised phenomenon (Bellamy and Hill 1998, McNicholl 1998). The more frequent diagnosis of mycobacterial infections in certain racial groups (Fine 1981) and results of twin studies (Shields *et al* 1987) suggest that host genotype plays a significant role in resistance to mycobacterial infections.

The set of MHC genes determines the repertoire of antigens the immune system of an individual is able to respond to, and thus is often linked to resistance or susceptibility to a particular disease. An association has been described between MHC antigens and two mycobacterial diseases, human leprosy (Todd *et al* 1990) and *M. a. intracellulare* pulmonary infection (Kubo *et al* 2000). Mutations and polymorphisms in a number of other genes have also been linked to susceptibility to mycobacterial disease. Patients with mutations in the gene coding for IFN-γ, the IFN-γ receptor (IFN-γR) or IL-12 (the main function of which is the induction of IFN-γ) have been found to exhibit greatly increased susceptibility to mycobacterial infections, even to normally avirulent, environmental species or attenuated vaccine strains (Altare *et al* 1998, Jouanguy *et al* 1999a, Jouanguy *et al* 1999b). The lepromatous, but not the tuberculoid form of leprosy, has been associated with a polymorphism in the promoter of the TNF gene (Roy *et al* 1997). Other genes reported to influence susceptibility to mycobacterial infections including NRAMP1 (Bellamy *et al* 1998)(see section 1.12.3 below), *sst1* (Kramnik *et al* 2000), and the genes influencing vitamin D (Bellamy *et al* 1999, Roy *et al* 1999, Gelder *et al* 2000).

1.12.2 Genetic resistance to infectious disease in animals

Some breeds of ruminants exhibit greater resistance to certain infectious diseases, for example indigenous African cattle breeds such as N'Dama and Baoule are more resistant to trypanosomiasis than imported breeds (Akol *et al* 1986). However, there are fewer instances where reported resistance to infectious disease in animals has been linked directly to certain genes or polymorphisms. Examples in sheep include an association between resistance to scrapie and certain PrP genotypes (Bossers *et al* 1997) and MHC haplotype and footrot, an infection caused by the synergistic action of *Dichelobacter nodosus* and *Fusobacterium necrophorum* (Escayg *et al* 1997). Macrophages from "resistant" cattle (classified on the basis of their response to natural *Brucella abortus* infection) were found to be more efficient than macrophages from "susceptible" cattle at controlling infection by *M. bovis* BCG, and *Salmonella dublin* (Qureshi *et al* 1996). This work is particularly relevant to the present study as it involves resistance to intracellular pathogens, suggesting that resistance to one also conferred resistance to others.

There have been few reports on the heritability of resistance to mycobacterial infections. A model of resistance to tuberculosis has been developed in deer in New Zealand by breeding resistant and susceptible lines of animals and comparing their responses to experimental *M. bovis* infection. Using this method, the estimated heritability of tuberculosis resistance in deer was calculated to be 0.48 (Mackintosh *et al* 2000). This is the first time that heritability of tuberculosis resistance in domestic livestock has been estimated and suggests that nearly half of the superior resistance to *M. bovis* infection demonstrated in selected deer is passed on to their offspring. A recent report suggested that marsupials are highly susceptible to mycobacterial infection and that this may be due to unique features of their immune system, which result in an inability to wall off infection sites (Buddle and Young 2000). This hypothesis suggests a genetic basis for the greater susceptibility of a whole class of animals to mycobacterial infection.

No specific genes have yet been identified that correlate with either the occurrence of paratuberculosis or the pathological changes seen in the disease in ruminants. However, anecdotal reports suggest that some strains and breeds of cattle and sheep are more susceptible to *M.a. paratuberculosis* (Withers 1959, Hole and Maclay 1959, Cranwell 1993), and genetic influences have been identified in inbred strains of mice infected with *M.a. paratuberculosis*

(Frelier *et al* 1990, Veazey *et al* 1995b). Recently, a standard polygenic statistical probit model was used to estimate the heritability of paratuberculosis in dairy cattle (Koets 2000). Using records from a 10 year vaccination trial, the heritability was estimated to be up to 0.09, considerably lower than the estimated heritability of deer to *M. bovis* (Mackintosh *et al* 2000). This level of heritability is comparable with other diseases, such as mastitis and ketosis. While low, the authors argue that substantial differences exist between animals in the extremes of the populations, thus suggesting selection of animals on the basis of their resistance to paratuberculosis could become part of management strategies for its control.

1.12.3 NRAMP1

Much research effort has been directed towards the function and importance of the highly conserved gene NRAMP1 which encodes a protein known as natural resistance associated macrophage protein (Nramp1). NRAMP1 (allelic with Ity/Lsh/Bcg) has long been suspected of playing a vital role in the phenotype of resistance or susceptibility to intracellular pathogens (Bellamy 1999). Nramp1 is found in the phagosome membrane of macrophages (Gruenheid *et al* 1997) and includes at least 10 hydrophobic, membrane-spanning domains. Its function and mechanism of action are still unknown but may include roles as a NO_2^- transporter (Vidal *et al* 1993) and iron metabolism and/or transportation (Gomes and Appelberg 1998, Zwilling *et al* 1999). *In vitro* experiments have found evidence that Nramp influences IFN-γ activation of macrophages (Radzioch *et al* 1994) and controls the intravacuolar environment of microbe-containing phagosomes (Gruenheid *et al* 1997). It has also been found to influence expression of major histocompatibility class II molecules on the surface of macrophages and regulate the processing of antigen by macrophages for presentation to $CD4^+$ T cells (Lang *et al* 1997). Information on the function of NRAMP1 has also been gained from analysis of related genes and their proteins. NRAMP2 is a similar gene, whose protein (Nramp2) is a transporter of bivalent cations (Gunshin *et al* 1997). Recently, a NRAMP homologue has been found in *M. tuberculosis* and *M. bovis* BCG (Agranoff *et al* 1999). This homologue has been termed MRAMP, and may compete with NRAMP1 for divalent cations in the hostile environment of the phagosome, with consequences for the intracellular survival of the invading organism.

A single base pair mutation in the NRAMP1 gene of certain inbred mouse strains - BALB/cJ, and C57BL/6J - has been identified (Vidal *et al* 1993). These mice exhibited increased susceptibility to several intracellular agents including *M.a. paratuberculosis*, *M. bovis* BCG, *M.*

lepraemurium, M. intracellulare, M. avium, Salmonella typhimurium, and *Leishmania donovanii* (Vidal *et al* 1995, Veazey *et al* 1995b, CanonneHergaux *et al* 1999). The base pair mutation resulted in an amino acid replacement, with a glycine residue being replaced by an aspartic acid at position 105 (Figure 1-6). This substitution occurred within one of the predicted transmembrane segments, and could therefore be expected to alter the physical properties of this transmembrane region significantly. This mutation has not been reported in any other species to date.

Figure 1-6. NRAMP1 mutation at position 105

Mouse^{r}	TTG G**G**C GGT leu gly gly
Mouse^{s}	TGG G**A**C GGT trp asp gly

Recently, evidence has been reported that suggests this mutation, while important, has only a limited effect on the resistance of the host to intracellular infections. For example the gly→asp substitution does not result in increased susceptibility of mice to virulent *M. tuberculosis* infection (Medina and North 1998) and mice functionally deleted of the NRAMP1 gene are as capable as wild type mice of resisting virulent *M. tuberculosis* infection (North *et al* 1999).

Conflicting data has also emerged from field work investigating the role of NRAMP1 in human disease. Polymorphisms in the NRAMP1 gene have been linked to susceptibility to tuberculosis (Bellamy *et al* 1998) and leprosy (Abel *et al* 1998), however, there have also been studies which have failed to find an association between the gene and susceptibility to leprosy (Roger *et al* 1997) or pulmonary *M. avium* infection in women (Huang *et al* 1998). These disparate results emphasise the complexity of host resistance to intracellular pathogens.

One study investigated the role of NRAMP1 in *M. bovis* infections in cattle (Barthel *et al* 2000). The authors examined 24 *M. bovis* infected ("susceptible") animals and nine uninfected ("resistant") and compared polymorphisms present in the 3′ untranslated region of the gene. No

associations were found between resistance or susceptibility to infection with *M. bovis* and polymorphisms in the NRAMP1 gene.

The anecdotal evidence of a genetic influence in resistance to paratuberculosis, combined with research suggesting a significant heritable component in other mycobacterial diseases, indicate the need for a thorough investigation into the genetic resistance and susceptibility of ruminants to *M.a. paratuberculosis.*

1.13 Treatment and Prevention

There is no effective treatment for paratuberculosis. Drugs such as streptomycin, isoniazid, and clofazimine have a transient effect at best (Chiodini *et al* 1984, Radostits *et al* 1997), and euthanasia is usually performed as soon as possible to minimise transmission of the disease. There are a number of management strategies recommended for reduction of levels of infection in cattle herds, most of these are aimed at minimising transmission of the disease to young calves, and identifying and culling infected animals as early as possible (Goodger *et al* 1996, Greig 2000). There are fewer recommendations for control of ovine paratuberculosis as many of the bovine strategies are impractical for a sheep flock.

There are vaccines available for paratuberculosis, however they do not prevent infection but may reduce the number of animals that develop clinical disease (Sigurdsson 1960, Doyle 1960, Garcia Marin *et al* 1997). Consequently, the number of organisms shed by infected animals is reduced, as is transmission of the disease. The efficacy of vaccination is unclear, with some workers recommending its use only in conjunction with management and husbandry changes. Use of the vaccine will result in positive responses to serological diagnostic tests, as well as interfere with tuberculosis tests (Stuart 1965, Spangler *et al* 1991).

1.14 Zoonotic potential

The possible link between *M.a. paratuberculosis* and Crohn's disease in humans has been extensively reviewed (Chiodini 1989, Thompson 1994, Vankruiningen 1999, Hubbard and Surawicz 1999). The two diseases have clinical similarities in that they both occur in young adults and both cause chronic enteritis, resulting in diarrhoea and weight loss. They are also both refractory to antibiotics and the histopathological changes of Crohn's disease bear some

similarities to the paucibacillary form of paratuberculosis. However, there are also differences since Crohn's disease affects the whole of the gastrointestinal tract, including the oral cavity and exhibits pathology such as ulceration, fibrosis, and fistulation, changes not associated with paratuberculosis (Scientific Committee on Animal Health and Animal Welfare 2000).

M.a. paratuberculosis is reported to have been cultured from a total of 10 patients with Crohn's disease, although there is an element of doubt concerning some of the reports (Vankruiningen 1999). There have also been numerous conflicting reports of detection and non-detection of IS900 in tissues and faeces of Crohn's patients, as well as other diseases, such as sarcoidosis, and inflammatory bowel disease. As there is great variation in the methods used in these reports, they are difficult to compare.

Recently, *M.a. paratuberculosis* has been isolated from pasteurised dairy cow's milk (Hoad 2000), and the breast milk of Crohn's disease patients (Naser *et al* 2000), suggesting this could be a means of transmission of the organism from mother to child, cow to calf, and cow to human. However, until *M.a. paratuberculosis* is proved to be the cause of Crohn's disease, this route, as a cause of disease in humans, remains merely speculative. The Report of the Scientific Committee on Animal Health and Animal Welfare (Scientific Committee on Animal Health and Animal Welfare 2000) concluded that "The current available evidence is insufficient to confirm or disprove that *M.a. paratuberculosis* is a causative agent of at least some cases of Crohn's disease in man".

1.15 Summary plan of thesis

The discovery of natural rabbit paratuberculosis in the Tayside region of Scotland has reignited debate into the role of sylvatic cycles of the organism. The aim of this thesis is to clarify the extent of natural non-ruminant wildlife infection with *M.a. paratuberculosis* and identify the effect these wildlife cycles have on the epidemiology of paratuberculosis. This will involve experimental infections to compare the virulence of isolates of the organism from different species, and investigation of possible transmission routes of *M.a. paratuberculosis* between wildlife and domestic livestock.

The existance of non-ruminant wildlife reservoirs of *M.a. paratuberculosis* suggests that vaccination, rather than the traditional detect and cull policies, may be the most effective means of control of the disease. However, currently available vaccines lack efficacy and produce significant side-effects. This thesis describes studies into the changes that occur during early paratuberculosis. A better understanding of host-pathogen interactions at this potentially crucial stage of infection could reveal how some animals sucessfully repel *M.a. paratuberculosis* infection, leading to the formulation of more accurately targeted and effective vaccines.

In the light of growing evidence of genetic susceptibility to a number of mycobacterial diseases, the role of host genotype in determining the susceptibility or resistance of an animal to paratuberculosis will be investigated. The effect of breeding for resistance to paratuberculosis has received little attention in the past but has the potential to become part of future control programmes, especially in affected sheep flocks.

2 Materials and Methods

2.1.1 Preparation of inoculum

To prepare a strain of *M.a. paratuberculosis* for use as inoculum, colonies were propagated on modified Middlebrook 7H11 solid media (see section 2.1.14 below), at 37°C for 8-10 weeks, then scraped off and resuspended in sterile phosphate buffered saline (PBS [170mM NaCl, 3.4mM KCl, 1mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.2]). An optical density reading, using a densimat (bio-Merieux) allowed an estimation to be made of the number of cfu ml^{-1}. The densimat estimates the bacterial density in a liquid medium, giving values in McFarland units. One McFarland unit approximates to $3x10^8$ cfu ml^{-1}.

The strains used throughout this thesis were *M.a. paratuberculosis* strain F13 (a bovine isolate), strain 51/91 (an ovine isolate), strain JD88/107 (a cervine isolate), and strain R7 (a rabbit isolate).

2.1.2 Culture of tissues, faeces, and urine

Tissue and faecal samples were cultured as described earlier (Greig *et al* 1999). A 1g sample of faeces or $0.5cm^3$ of tissue was homogenised for 30 seconds in 10ml sterile distilled water using a Colworth Stomacher 80 (Seward Medical). The homogenates were then decontaminated by adding 10ml of 1.5% [v/v] hexadecyl pyridinium chloride (Sigma-Aldrich) and left to stand overnight at room temperature. The supernatants were centrifuged at 3 800 x g for 30 min at 4°C and each pellet resuspended in 10ml of sterile distilled water. The centrifugation step was repeated, and each pellet resuspended in 1ml of sterile distilled water. The suspension was then transferred to a microcentrifuge tube and centrifuged at 6 500 x g for 5 minutes at room temperature and the pellet resuspended in 0.5ml of sterile distilled water. 0.1ml of this suspension was then inoculated onto two slopes of modified Middlebrook 7H11 agar (section 2.1.14 below). The cultures were incubated at 37°C for up to 16 weeks and examined regularly for bacterial growth.

Urine samples were centrifuged at 3 800 x g for 30 minutes at room temperature, and the deposit resuspended in 10ml 0.75% [v/v] hexadecyl pyridinium chloride. This was left to stand at room temperature overnight, then the supernatant processed as for faeces and tissue.

2.1.3 Extraction of DNA from mycobacterial colonies

A 200µl aliquot of sterile distilled water was inoculated with a single bacterial colony from a culture. This was then added to a screw-capped eppendorf tube containing 1ml of zirconium beads (0.1mm diameter) (BioSpec Products), and cooled on ice. The tube was placed in a Hybaid ribolyser (Hybaid), and ribolysed at 5.5 $m.s^{-1}$ for 20 seconds, and then cooled on ice for one minute. The DNA was extracted using guanidine hydrochloride as described previously (Challans *et al* 1994), then screened for the presence of IS900 using a PCR-ELISA microplate assay (section 2.1.15 below).

2.1.4 Retrospective viable count method

Ten-fold serial dilutions were made from the inoculum (1 to 10^{-6}), with 100µl of each dilution plated (in duplicate) onto modified Middlebrook 7H11 media (section 2.1.14 below), and incubated at 37°C. After six weeks incubation, the number of colonies on the slopes were counted, and an average calculated for the number of cfu ml^{-1} of the original inoculum.

2.1.5 Preparation of histological sections

Samples were fixed in 10% [v/v] formal saline for a minimum of 24 hours, then trimmed, dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin wax. Sections 5µm thick were cut and stained with H&E for routine histopathological examination, and for AFB by the ZN method.

2.1.6 Ziehl-Neelsen stain

Sections were dewaxed in xylene, and hydrated through graded alcohols to water. Each slide was then flooded with filtered carbol fuchsin (containing 1% [w/v] basic fuchsin, 10 % [v/v] absolute alcohol and 0.5% [v/v] phenol), and left at 37°C for at least 1 hour, or 60°C for 30 minutes. They were then rinsed in tap water and decolourised with acid alcohol (70% [v/v] methylated spirits, 1% [v/v] hydrochloric acid) until the section became pale pink. They were then rinsed in running tap water, counterstained briefly with methylene blue solution (0.25% [w/v] methylene blue in 1% [v/v] acetic alcohol), differentiated in 70% [v/v] alcohol, dehydrated, cleared and mounted in DPX mounting medium (BDH).

2.1.7 Ziehl-Neelsen smear

A thin smear of a representative piece of tissue or sample of faeces was made on a slide, and allowed to dry. It was then flooded with filtered carbol fuchsin (see section 2.1.6 above), heated gently to steaming and allowed to steam for 5 minutes. After washing in tap water, the slide was decolourised with acid alcohol (see section 2.1.6 above) for 1-2 minutes and washed again in tap water. The slide was then counterstained with methylene blue solution (see section 2.1.6 above) and washed in tap water.

2.1.8 Schmorl's reaction to detect lipofuscin

Sections were dewaxed in xylene, hydrated through graded alcohols to water, and then immersed in freshly prepared incubating solution (0.75% [w/v] ferric chloride, 0.1% [w/v] pottasium ferricyanide) for 30 seconds to five minutes. After rinsing in running tap water, the sections were treated with 1% [v/v] acetic acid for five minutes, counterstained in 1% [w/v] neutral red (BDH) for two to five minutes, washed in tap water again, and finally dehydrated through graded alcohols to xylene and mounted.

2.1.9 Immunohistochemistry

Cryostat sections 6μm-thick were cut from the frozen tissues onto treated slides (Vectabond® [Vector Laboratories] or Superfrost® plus [BDH]), air dried overnight, fixed for 10 minutes in acetone (Sigma-Aldrich), and stored at -20^{0}C. Labelling was carried out using an indirect immunoperoxidase technique with a commercially available kit (Vectastain Elite ABC kit, Vector Laboratories). Manufacturers instructions were followed. Slides were rehydrated for 10 minutes in a blocking buffer (2% [v/v] normal sheep serum [Scottish Antibody Production Unit] in PBS), and then incubated in 0.3% [v/v] hydrogen peroxide diluted in 100% methanol for 30 minutes at room temperature. Incubation with primary antibody was carried out for one hour at room temperature. Visualisation of bound antibody-HRP complex was performed using a DAB substrate kit (Vector Laboratories), according to the manufacturers instructions. Following colour development (2-5 minutes) sections were washed in tap water, lightly counterstained in haematoxylin, dehydrated through graded alcohols and mounted in DPX mounting medium (BDH).

2.1.10 Fluorescent antibody cell sorting (FACS) method

Lymphocytes were isolated from peripheral blood, JPP, IPP, and MLN for FACS analysis. Blood samples were taken immediately prior to euthanasia by jugular venipuncture into sterile 10ml "vacutainer" tubes (Becton-Dickinson) containing 10 IU of preservative-free heparin (Sigma-Aldrich). 10ml of heparinised whole blood was layered on 10ml "Lymphoprep" (Nycomed) and centrifuged at 1000 x g for 30 minutes at room temperature. Plasma was removed and stored at –20^{o}C for possible later use. The band of lymphocytes was removed into 10ml sterile wash medium (Ca^{2+}, Mg^{2+} free HBSS [Life Technologies] containing 100 units ml^{-1} penicillin, 0.1mg ml^{-1} streptomycin, 20 units ml^{-1} gentamicin and 1% [w/v] amphotericin B) and washed twice by centrifugation at 300 x g for 10 minutes. The final pellet was resuspended in 1ml of HBSS and the lymphocytes counted and the concentration adjusted to $1x10^{7}$ cells ml^{-1} for FACS analysis.

The MLN, JPP and IPP lymphocytes were collected as follows: the JPP, IPP and MLN were collected into sterile wash medium. Tissues were then trimmed of fat, homogenised in a Colworth Stomacher 80 (Sewell Medical) and more wash medium added. Cells were then filtered through two pieces of lens tissue and washed twice with centrifugation at 300 x g for 10 minutes. Cell counts were adjusted to $1x10^{7}$cells ml^{-1} in wash medium for FACS analysis.

Single colour flow cytometric analysis was performed on lymphocytes as previously described (Begara-McGorum *et al* 1998). Briefly, 100µl of each cell suspension ($1x10^{7}$ cells ml^{-1}) was incubated with 50µl of each monoclonal antibody for 30 minutes at 4^{o}C and washed twice with PBA (PBS, 1% [w/v] bovine serum albumin [BSA], 0.01% [w/v] sodium azide). 50µl of a 1/100 dilution of FITC-conjugated rabbit anti-mouse immunologlobulin (Dako) was added and the incubation repeated. After two further washes in PBA, cells were fixed in a solution of 4% paraformaldehyde and stored at 4°C in the dark until analysis on a FACScan flow cytometer (Becton Dickinson). Results were expressed as a percentage of positively stained cells in a sample population of 10 000 individual cells.

2.1.11 Formalin-fixed paraffin-embedded (ffpe) tissue PCR

To minimize the chance of contamination during cutting of the wax blocks, the work bench and microtome were thoroughly cleaned with 100% ethanol prior to sample collection, and between

sampling of each block. A negative control block of tissue was also included after every five sample blocks cut, and sterile equipment used for each block. Each block was wiped with 100% ethanol before cutting. One 10μm-thick section was cut from each block and sealed in a sterile tube. Subsequently, the wax was dissolved using a xylene extraction method. Briefly, 1200μl of xylene was added to each tube, and vortexed vigorously. After centrifugation at 13 000 x g for five minutes at room temperature, the supernatant was removed. The process was repeated twice using 1200μl ethanol. The pellet was incubated at 37°C for 30 minutes or until the remaining ethanol evaporated from the tube. The pellet was resuspended in 80μl of PBS and transferred to a microcentrifuge tube containing zirconium beads (BioSpec Products). The sample was ribolysed (Hybaid Ribolyser, Hybaid) at $5.5m.s^{-1}$ for 20 seconds, then cooled on ice. The supernatant was transferred to a fresh microcentrifuge tube. The DNA was then extracted using the DNeasy Tissue Kit (Qiagen), following the manufacturers instructions. 5μl of the DNA was tested by the IS900 PCR (section 2.1.15).

2.1.12 Detection of PCR product using silver-stained polyacrylamide gels

A 7.5% continuous polyacrylamide gel (containing 1 x TBE [0.89 Tris, 0.89M boric acid, 0.025M Na_2EDTA], 0.05% [w/v] ammonium persulfate, 0.1% [v/v] temed and 25% [v/v] acrylamide solution (Scotlabs) [30% [w/v] acrylamide, 0.8% [w/v] NN-methylene bis-acrylamide, ratio 37.5:1]) was poured in a Bio-Rad mini-PROTEAN II gel apparatus, and allowed to stand for a minimum of 30 minutes. 5-20μl of sample, with 2μl loading dye (Promega UK) was then run in 1 x TBE buffer for 30 minutes at 200V.

After electrophoresis, gels were stained using silver nitrate. Gels were soaked for 10 minutes in a solution of 10% [v/v] ethanol and 0.5%[v/v] acetic acid, then gently agitated in a solution of freshly made 6mM silver nitrate. After rinsing briefly in distilled water, the bands were developed by adding freshly made developer solution (0.28M sodium hydroxide containing 0.05% [v/v] formaldehyde 37-42% solution) until the desired level of staining was obtained. The reaction was stopped by adding a 0.75% [w/v] solution of sodium carbonate. Typically, 100ml of each solution were used at each stage.

2.1.13 Agarose gel for seperating ffpe fragments

A 2% [w/v] agarose gel in 0.5 x TBE was poured into gel mould, and, once cool, 5-20µl of sample loaded. After being run in a 0.5 x TBE buffer for 1.5-2hr at 70V to separate the DNA fragments, it was then post stained in 0.01% [w/v] ethidium bromide before being viewed on a transilluminator.

2.1.14 Middlebrooks modified media

The medium was made up with 2.1% [w/v] 7H11 agar (Difco), 2.3mM L-asparagine, 2.5% [v/v] glycerol, and 2µg.ml^{-1} mycobactin J (Allied Monitor). This was sterilised at 121^{o}C for 15 minutes, then supplemented with 10% [v/v] Middlebrook OADC enrichment (Difco), 20% [v/v] heat-inactivated new born calf serum (Life Technologies), and two Selectatabs® (amphotericin B, polymixin B, carbenicillin, and trimethoprim; MAST laboratories) per litre of media.

2.1.15 Method for IS900 PCR and ELISA

This method was developed by Karen Stevenson, Paul Brooks, Karen Rudge, Tom Brown and J. Michael Sharp.

2.1.15.1 Oligonucleotide primers and detection probe

The oligonucleotide sequences and modifications are given in Table 2-1. The primers (90 and 91) amplified a 389 bp fragment of the IS900 sequence. Primer 91 was labelled with a 5' biotin molecule. The detection probe for the ELISA (probe C2297) was an oligonucleotide with a sequence complementary to the region amplified by primers 90 and 91, labelled with a 5' triple dinitrophenyl (DNP) molecule. All oligonucleotides were synthesised by Oswel Ltd.

Table 2-1 Oligonucleotide primers and probes used in method 2.1.15

Primer/probe	Nucleotide sequence (5'-3')	Modification
Primer 90	GTTCGGGGCCGTCGCTTAGG	None
Primer 91 BIT	CCCACGTGACCTCGCCTCCA	5'- Biotin
Probe C2297	GGGTTGATCTGGACAATGAC	5'- Triple DNP

2.1.15.2 Amplification by PCR

Each reaction was performed in a final volume of 50µl containing 1 x Promega PCR buffer (Promega UK), 2mM $MgCl_2$, 0.5µM of each primer, 250µM of each dNTP and 1U *Taq*

polymerase (Promega UK). Five microlitres of extracted DNA was added to each reaction. The reactions were carried out in a Hybaid Touchdown PCR thermocycler in polycarbonate 96-well microplates, conically welled, with a capacity of 0.2ml (Omniplate 96, Hybaid), or 0.2ml Micro-strips (ABgene), and sealed with adhesive plate seals (ABgene). After an initial hot start at 95^{0}C for 5 min to denature the DNA, the reaction parameters were denaturation at 95^{0}C for 1 min, annealing at 55^{0}C for 1 min and extension at 72^{0}C for 1 min, for 40 cycles, followed by a final extension step at 72^{0}C for 3 min.

2.1.15.3 Detection of PCR product by ELISA

Following amplification by PCR, 5µl (5µM) of detection probe C2297 was added to each reaction. The tubes or plate were then resealed and the samples heated at 95°C for 5 minutes. After cooling at room temperature for a minimum of 15 minutes, each sample was added to a well of the streptavidin-coated microtitre plate (Boehringer Mannheim), which contained 50µl of a solution of 0.1% BSA in PBS. Each sample was mixed, the plate sealed, and incubated at room temperature for 30 minutes. The samples were then removed and discarded and the wells washed three times with 300µl of PBS 0.1% BSA, including a 2 minute soak between washings. One hundred microlitres of a 1:2000 dilution of peroxidase-conjugated rabbit anti-DNP (Dako) in PBS 0.1% BSA were added to each well, the plates were resealed and incubated at room temperature for 30 minutes. The antibody solution then was removed and the wells washed three times with 300µl of PBS 0.1% BSA, with a 2 minute soak between washings. For colourimetric detection, 200µl of a 2.2mM solution of O-phenylenediamine dihydrochloride (OPD) was added to each well and the samples incubated for 15 min at room temperature. The reaction then was stopped with the addition of 50µl of 2M HCl. The OD_{490} readings were obtained using a Dynatech MR5000 microplate reader (Dynex Technologies).

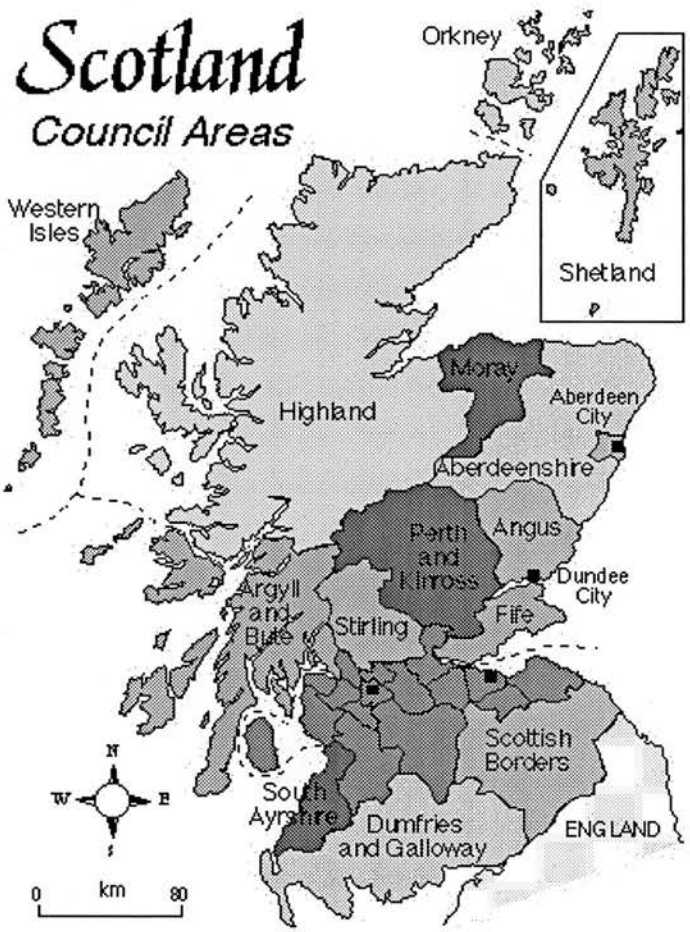
3 Characterisation of Paratuberculosis in Rabbits

3.1 Introduction

In the past it has been assumed that natural paratuberculosis occurred only in ruminants, despite the susceptibility of a number of non-ruminant species to experimental *M.a. paratuberculosis* challenge (see section 1.11 above). Consequently, control and eradication programmes considered only ruminant reservoirs of the organism. However, in 1990, paratuberculosis-like lesions were reported in a rabbit from a paratuberculosis-affected farm (Angus 1990). Subsequent investigations have identified a high incidence of natural *M.a. paratuberculosis* infection of rabbits in the Tayside region of Scotland, and identified a statistically significant relationship between farms with a history of bovine paratuberculosis, and the presence of the disease in the rabbit population (Greig *et al* 1997, Greig *et al* 1999). This previous work therefore reveals that *M.a. paratuberculosis* has the ability to infect non-ruminant, as well as ruminant wildlife species, and suggests that the cycles of the organism in rabbits and cattle are linked.

This chapter describes the pathological features of natural paratuberculosis in rabbits and compares and contrasts these features to those of experimental rabbit and naturally occurring ruminant paratuberculosis. The suitability of four techniques for the diagnosis of natural rabbit paratuberculosis are investigated and the potential for rabbits to transmit the disease to domestic ruminants discussed.

Figure 3-1 Map of Scotland, depicting the administrative regions*.



*Angus, Perth and Kinross regions were grouped together as the Tayside region until 1994.

3.2 Materials and Methods

3.2.1 Collection of rabbits.

As part of the normal rabbit control programme, 110 rabbits were collected from seven farms on which paratuberculosis had previously been identified as causing clinical disease in livestock (Table 3-1). Farms A, C and D were in Angus, farms B, E and F in the Perth and Kinross region, while farm G was located in the Highlands (Figure 3-1).

Rabbit carcasses were collected from each farm, and examined for any sign of disease or ill health before the abdomen was opened, and samples collected for histopathological and microbiological examination.

Table 3-1. Comparison of the prevalence of rabbit paratuberculosis on each of the seven farms involved in the study.

Farm	domestic ruminants affected by paratuberculosis[1]	number of rabbits collected	number of rabbits positive[2]	prevalence of infected rabbits (%)
A	cattle	23	5	22%
B	cattle sheep suspect[3]	26	4	15%
C	adjacent to an affected cattle farm	9	1	11%
D	cattle sheep	20	5	25%
E	cattle sheep suspect[3]	11	2	18%
F	cattle	14	6	43%
G	deer	7	1	14%
TOTAL		**110**	**24**	**22%**

[1]Paratuberculosis diagnosed by culture and/or histopathology of samples from clinically affected domestic ruminants

[2]Positive by any of the following diagnostic methods: culture of tissue, culture of faeces, histopathology, ffpe PCR.

[3]Farms B and E reported a history of ill-thrift in sheep, but no definitive diagnosis of paratuberculosis had been made.

3.2.2 Microbiological examination

An impression smear was made from a sample of duodenum, ileum, sacculus rotundus and MLN using a ZN stain (section 2.1.7 above). A pool of tissues (duodenum, ileum, sacculus rotundus and MLN) and faeces from rabbits that showed AFB in the impression smear were cultured separately to confirm the identity of the AFB. Urine was cultured from 38 rabbits which had a sufficient quantity of urine for collection. Tissues, faeces and urine were cultured on modified Middlebrooks 7H11 medium and any isolates recovered examined by PCR for the IS900 insertion sequence (described in sections 2.1.2, 2.1.3, and 2.1.15).

3.2.3 Histopathological examination

Samples of small intestine, sacculus rotundus, caecum, appendix, large intestine, MLN and liver were taken for histopathological evaluation (see Plate **5,10**). Sections were processed and stained by H&E and ZN methods as described previously (see sections 2.1.5 and 2.1.6 above). Selected sections containing unidentified brown pigment were stained with Schmorl's method (see section 2.1.8 above) to identify lipofuscin. The extent and severity of lesions in the tissues were assessed and graded as mild or severe. Mild changes were defined as histopathology consisting of fewer than approximately 10 small granulomata in total, with sparse intracellular AFB. Samples with extensive infiltration by chronic inflammatory cells, numerous large granulomata, and abundant intracellular AFB were regarded as severe.

3.2.4 Immunohistochemistry

Samples of small intestine and MLN were collected at the time of necropsy, snap frozen in a slurry of dry ice and isopentane for approximately 30 seconds, and stored at -70°C until required. A monoclonal antibody raised against *M.a. paratuberculosis*, that recognizes a 65kDa protein in a number of mycobacterial species (K. Stevenson, unpublished data) was used as primary antibody (see section 2.1.9 above).

3.2.5 Formalin-fixed paraffin-embedded (ffpe) tissue PCR

The technique is described in section 2.1.11.

3.3 Results

A total of 24 out of 110 rabbits examined (22%) were positive by one or more methods (Table 3-2).

3.3.1 Microbiological examination

Smears from 17 of the 110 rabbits revealed AFB when stained with the ZN method, consequently, faeces from these 17 rabbits, and pooled tissues from 15 of the 17, were cultured. *Mycobacterium avium* subsp. *paratuberculosis* was isolated from all these samples, and from the urine of two rabbits (Table 3-2).

3.3.2 Pathology

No gross pathological changes were noted in any of the rabbits but in 18 of the 98 rabbits available for histopathological examination (18.4%) there were lesions consistent with *M.a. paratuberculosis* infection.

3.3.2.1 Intestines

Thirteen of the 18 rabbits with histopathological changes had lesions present in the intestines (Table 3-3) and in ten cases they were considered to be severe, characterised by infiltration of the villous lamina propria of the small intestine by large numbers of epithelioid macrophages and giant cells (Plates **3,1-3,4**). This infiltrate was associated with a marked attenuation of villous architecture including stunting and broadening of the villi. The giant cells were a prominent feature of the lesions, some containing as many as 50 nuclei. The epithelial surface appeared normal. Foci of epithelioid cells containing AFB extended from the lamina propria through the submucosa and into the intestinal serosa (Plates **3,5** and **3,6**). Small numbers of macrophages containing AFB were identified in the villi of the colon of four of the 10 rabbits (Plate **3,9**).

Three of the 13 rabbits with histopathological lesions in the intestines exhibited mild changes, consisting of small aggregates of macrophage-like cells at the base of the lamina propria in the small intestine (Plates **3,7** and **3,8**). Small numbers of AFB were found in a variable proportion of these cells.

Table 3-2. A comparison of the results from the 24 rabbits which had evidence of *M.a. paratuberculosis* infection, showing the relationship between severe histopathology and positive ZN smear, culture and ffpe results.

Rabbit number	Sex	ZN smear	Culture			Histopathology grade[c]	ffpe
			Tissue	Faeces	Urine		
1	f	+	+	+	-	S	+
2	f	+	+	+	-	-	-
3	m	+	+	-	-	-	-
4	f	+	+	+	na	S	-
5	m	+	+	+	-	M	-
6	m	+	+	+	-	S	-
7	m	+	+	+	+	na	na
8	f	+	+	-	na	na	na
9	m	+	+	+	na	na	na
10	f	+	+	+	-	S	+
11	m	+	+	+	-	S	-
12	m	+	+	+	+	S	+
13	f	+	+	+	-	S	-
14[a]	f	+	+	+	-	-	-
15	f	+	+	+	na	S	+
16	m	-	na	na	na	M	-
17	m	-	na	na	-	M	+
18	m	-	na	na	na	M	+
19	m	-	na	na	na	M	-
20[b]	f	+	na	+	na	S	+
21	m	+	na	+	-	S	+
22	m	-	na	na	na	M	-
23	m	-	na	na	na	M	-
24	f	-	na	na	na	M	-
Total number positive		**17**	**15**	**15**	**2**	**18**	**8**

na: procedure not attempted.
[a]juvenile
[b]pregnant
[c]lesions were graded as none (-), mild (M) or severe (S) (see section 3.2.3 above)

3.3.2.2 Gut associated lymphoid tissue (GALT)

In the ten rabbits with severely affected intestines, numerous epithelioid cells containing abundant AFB were seen in the small lymphoid patches underlying the lamina propria of the small intestine (Plate **3,5**). There were also aggregates of epithelioid macrophages, containing numerous AFB, in the sacculus rotundus and the appendix (Plate **3,10-12**). These aggregates were most often found in the interfollicular zone at the base of the lymphoid follicles. A smaller number of epithelioid and giant cells, with intracellular AFB, were observed in the villi overlying these lymphoid areas. The proximal portion of the caecum appeared normal. No lesions were noted in the GALT of the remaining eight rabbits with histopathological changes.

3.3.2.3 Mesenteric lymph node

The ten rabbits with severe lesions in the small intestine and GALT also exhibited extensive changes in the MLN (Plate **3,17**)(Table 3-3). A large number of epithelioid and giant cells, containing numerous AFB in their distended cytoplasm, were observed in the cortex of these lymph nodes (Plates **3,13** and **3,14**). A few similar cells were also seen in the medullae of these MLN. A further six rabbits showed more subtle pathology, composed of small numbers of chronic inflammatory cells, sometimes aggregated to form small granulomata, with very few AFB present. In one rabbit (number 22, Table 3-2), no lesions were found in the intestinal tissues but large numbers of epithelioid and giant cells were present in the cortex of the MLN. Only a small number of intracellular AFB were found in the MLN in this case (Plates **3,15** and **3,16**).

Table 3-3. The distribution and extent of histopathological changes noted in the intestines and MLN of each affected rabbit.

		tissue affected			
Grade of lesion		**intestine only**	**MLN only**	**intestine and MLN**	**TOTAL**
	Mild	2	5	1	8
	Severe	0	0	10	10
					18

3.3.2.4 Liver

All ten rabbits with severe lesions in the intestines, GALT and MLN also exhibited hepatic pathology, consisting of multiple foci of granulomatous inflammation (Plate **3,18**). The foci were predominantly located in the periportal areas and consisted of admixed lymphocytes, macrophages, and multinucleate giant cells. The latter two cell types frequently contained AFB, which were also evident in a small number of scattered Kupffer cells. No granulomatous lesions were noted in the liver of the mildly affected rabbits.

3.3.2.5 Other pathological findings.

Evidence of enteric coccidiosis was found in 67% of rabbits, and lesions consistent with hepatic coccidiosis noted in 18% (Plates **3,19** and **3,20**). In one case there were hepatic changes consistent with liver fluke infestation (Plate **3,28**). The presence of unidentified intestinal worms was a relatively common finding (35% of rabbits) (Plates **3,21** and **3,22**). None of the intestinal worms examined had AFB present in their gut, or adherent to their surfaces. A number of rabbits (some with and some without paratuberculosis) had, in the MLN and intestinal lymphoid areas, aggregates of large macrophages containing numerous yellow granules in the cytoplasm. These granules were ZN negative, but positive for lipofuscin when examined with Schmorl's stain (Plates **3,23-3,25**).

3.3.3 Immunohistochemistry

Immunohistochemistry was carried out on samples of gut and MLN both with and without lesions of paratuberculosis. The results mirrored those of the ZN stained sections, with macrophages and giant cells that contained AFB on ZN stained sections also labelling positive with the immunohistochemical procedure. No positive labelling was identified on the sections that were classified negative by histopathology (Plates **3,26** and **3,27**).

3.3.4 Formalin-fixed paraffin-embedded (ffpe) tissue PCR

The PCR was performed on ffpe tissues from 32 rabbits (Table 3-4). Of these, 11 were negative by histopathology, and 21 were positive by histopathology, microbiology, or both. Using the ffpe PCR, eight of the 32 rabbits were positive, and six of these had severe histopathological lesions with large numbers of AFB present. The remaining two positive rabbits had mild lesions, with only small numbers of AFB noted on histopathological examination (Table 3-2). All 11

rabbits that were negative on histopathological and microbiological examination were also negative by the ffpe PCR, as were the negative control sections.

Table 3-4 A comparison of the results from the ffpe PCR and the microbiological and histopathology examination of tissues.

	result of histopathological and microbiological examination:		Total
	positive[1]	negative[2]	
ffpePCR +	8	0	**8**
ffpe PCR -	13	11	**24**
Total	**21**	**11**	**32**

[1]positive by histopathological and/or microbiological examination

[2]negative by both histopathological or microbiological examination

3.4 Discussion

Evidence of infection by *M.a. paratuberculosis* was found in 22% of rabbits examined from seven farms in Scotland, and confirms the presence of the organism in free-living rabbits. Histopathological lesions were either mild or severe, with the most extensive changes found in the small intestines and MLN. The histopathological changes were distinct from those described in ruminant cases of paratuberculosis, but similar to those reported in experimental cases of rabbit paratuberculosis.

3.4.1 Histopathological lesions noted in affected rabbits

No gross lesions suggestive of *M.a. paratuberculosis* infection were found in any of the 110 rabbits examined. This is in contrast to earlier studies in which thickened gut and swollen lymph nodes were reported in a small number of affected rabbits (Angus 1990, Greig *et al* 1997). The lack of gross lesions in the severely affected rabbits is surprising, considering the marked histopathological changes noted, however lack of correlation between gross changes, clinical signs, and the extent of histopathological lesions is reported as a feature of ruminant paratuberculosis (see section 1.6 above).

Histopathological examination in this study showed that ten rabbits had severe intestinal lesions and each also exhibited extensive changes in the MLN, as well as the liver. The remaining eight rabbits with histopathological lesions had mild changes in their intestines and/or MLN and no detectable paratuberculosis-like lesions in the liver. The consistency of the pathology in the tissues of each rabbit allowed it to be classified either as mild or severe.

The lesions noted in the affected rabbits were compared to the changes associated with *M.a. paratuberculosis* infection of ruminants. The histopathological changes associated with the subclinical period of ruminant paratuberculosis consist of discrete aggregates of macrophages containing small numbers of AFB in the lymphoid tissue and, sometimes, the lamina propria of the intestine, while in clinical paratuberculosis the pathological changes are classified as either paucibacillary or multibacillary. The intestines of animals with multibacillary disease are infiltrated with large numbers of swollen, foamy macrophages with abundant intracellular AFB, while the paucibacillary form of pathology is characterised by increased numbers of lymphoid cells, occasional giant cells, and very few, if any, AFB (Buergelt *et al* 1978, Clarke and Little 1996, Perez *et al* 1996)(see section 1.6 above). Therefore, while the histopathological changes seen in the mild form of rabbit paratuberculosis correlate with those described in subclinically affected ruminants, the changes in severely affected rabbits are distinct from both the multibacillary and paucibacillary forms of ruminant paratuberculosis. The lack of lymphocytic inflammation and the abundance of AFB precludes its classification as paucibacillary, while the presence of numerous giant cells differentiates it from multibacillary paratuberculosis. The reasons for the development of unique lesions in severe cases of rabbit paratuberculosis are unknown, although giant cells have been reported to occur in other leporine diseases, including *M. avium* infection (Himes *et al* 1989), malignant cattarhal fever (Buxton *et al* 1984), and myxomatosis (Marcato 1974), suggesting that they may occur in rabbits in response to a variety of diseases.

The mildly affected rabbits showed little if any change in the MLN, with one exception. No AFB were seen in the ZN smear of tissues from rabbit 22 (Table 3-2), therefore culture of samples from this rabbit was not attempted. No histopathological lesions were found in intestinal tissues, but large numbers of epithelioid and giant cells were noted in the cortex of the MLN, and a small number of intracellular AFB were found in a minority of these cells (Plates **3,15** and **3,16**). A similar case was described in a previous survey (Greig *et al* 1997) - a rabbit was reported with

giant cells scattered throughout the lymph nodes, but no acid fast bacilli were detected. A mycobacterium from the *M. smegmatis - M. phlei* group was cultured from the intestines of this rabbit. The unusual pathology seen in rabbit 22 suggests that the organisms identified may be another species of mycobacterium, possibly from the above group.

The presence of macrophages in the lymphoid tissue of the sacculus rotundus and appendix has previously been described as indicative of infection by *M.a. paratuberculosis* (Greig *et al* 1997). However, in this survey small accumulations of lipofuscin-positive, AFB-negative macrophages were identified in the lymphoid tissue of many rabbits, both culture positive and negative (Plates **3,23-3,25**). Lipofuscin is an intralysosomal pigment formed by the peroxidation and polymerisation of unsaturated fatty acids (Jones *et al* 1997). In a study investigating the susceptibility of rabbits to experimental inoculation with *M. bovis*, similar lipofuscin-positive, AFB-negative macrophages were described surrounding extensive granulomatous pulmonary lesions (Converse *et al* 1996). Examination of the lymphoid tissue of specific pathogen free (SPF) rabbits has also revealed the presence of similar macrophages (Plate **3,29**). This suggests that small clusters of macrophages in the lymphoid tissue of rabbits are a non-specific and probably normal finding, and that the presence of AFB is necessary for designating a lesion consistent with *M.a. paratuberculosis* infection. Identification of intracellular AFB was a prerequisite for classifying samples as histopathology positive in the free-living rabbits examined in this survey.

Hypotheses regarding the pathogenesis of rabbit paratuberculosis may be drawn from the pathological descriptions of the disease. The histopathological changes noted in the rabbits suggest that, like ruminants, they undergo a subclinical period of infection, corresponding to the mild pathological changes recorded in this chapter, which may then progress to severe lesions and clinical disease. However, the length of the subclinical period and the factors influencing a transition to severe disease are unclear. Alternatively, it is possible that some rabbits are innately more susceptible to *M.a. paratuberculosis* so that while the resistant animals develop the mild form of the disease, the susceptible rabbits are less able to inhibit multiplication of the organism and consequently develop extensive and severe histopathological changes.

3.4.2 Mycobacterial infection of free-living lagomorphs

There are few reports of natural mycobacterial infection of lagomorphs. *Mycobacterium bovis* infection of a small number of wild rabbits and hares has been described in New Zealand (Anon. 1980, Cooke *et al* 1993) as well as a suspected *M.a. paratuberculosis* isolation from a hare in southern England (Matthews and Sargent 1977). This latter study examined tissues from 285 hares (*Lepus europaeus*) and cultured mycobacterial species from five. Four of the five isolates were identified as *M. avium* type 2, but the fifth, while showing features consistent with *M.a. paratuberculosis*, was never conclusively classified.

3.4.3 Experimental paratuberculosis in rabbits

There are numerous reports of experimental infection of rabbits with *M.a. paratuberculosis*. Early workers (Hirch 1956, Harding 1959) reported slight lesions in a minority of orally inoculated rabbits, but did not provide full pathological descriptions. More recently, paratuberculosis-like lesions were induced in 62% of newborn rabbits after an incubation period of 11 months (Mokresh *et al* 1989, Mokresh and Butler 1990). No gross lesions suggestive of paratuberculosis were noted on post mortem examination, although some of the rabbits had experienced intermittent periods of diarrhoea during the incubation period, and one rabbit later diagnosed with advanced paratuberculosis exhibited severe emaciation. The lesions described in the experimentally infected rabbits consisted of "granulomas consisting mainly of macrophages" in the appendix and sacculus rotundus. The macrophages had variable numbers of AFB in their cytoplasm. As sections from other areas of the gastrointestinal tract were not extensively examined, a full comparison cannot be made with the lesions described in free-living rabbits.

3.4.4 Diagnosis of rabbit paratuberculosis

Use of the PCR on ffpe tissues has been described for the diagnosis of a number of mycobacterial infections (Plante *et al* 1996, Vago *et al* 1998, Whittington *et al* 1999a). In this survey, it identified eight positive samples, six of which were from severely affected rabbits. The procedure was used to confirm the diagnosis in two samples for which culture from fresh tissue was not performed (Table 3-2, rabbits 20 and 21). The length of time the tissue is fixed for, the amount of template DNA in the sample, and the length of the target sequence are three factors known to influence the success of the ffpe PCR technique.

Formalin fixation is known to both fragment and cross link DNA (Koshiba *et al* 1993), with some studies detecting an inhibition of the PCR reaction after only 24 hours in formalin fixative (Benezra *et al* 1991). Due to transportation restrictions, some of the samples in this study remained in formalin fixative for up to three weeks, thus decreasing the ability of the PCR to detect mycobacterial DNA.

The ffpe PCR results reported here reveal a correlation between severe histological lesions with high numbers of AFB present and a positive ffpe PCR result, indicating the necessity for an adequate amount of template in the sample (Table 3-2). A low number of template copies of DNA present in the sample has been found to be a significant restricting factor in other investigations utilising PCR to detect mycobacterial DNA from ffpe tissues, with sensitivity as low as 12.5% reported in one study (Plante *et al* 1996, Whittington *et al* 1999a).

Some workers recommend PCR targets of between 80 and 170bp in length (Shibata 1994), while a study amplifying DNA from *M.a. paratuberculosis* in the tissues of paratuberculosis affected ruminants revealed increased sensitivity when targeting a 229bp rather than a 413bp fragment (Whittington *et al* 1999a). The PCR fragment in the protocol described here was 389bp long, consequently, targeting a smaller fragment may therefore result in increased sensitivity of the ffpe PCR procedure described in this study.

Culture of *M.a. paratuberculosis*, followed by the use of PCR to confirm the identity of the isolate, can take up to four months, and has the inherent problems of contamination and overgrowth of mycobacterial colonies. By comparison, applying the PCR to ffpe tissues can be completed in two days, does not require prior growth of colonies, and may be a useful retrospective tool to complement histopathological examination in low budget surveys of wildlife for paratuberculosis infection.

Immunohistochemistry was performed on samples of rabbit tissues to determine if it was more sensitive at detecting the *M.a. paratuberculosis* organism when compared with the use of a ZN stain. No antigen has yet been identified that is 100% specific for *M.a. paratuberculosis* (Stevenson and Sharp 1997), consequently immunohistochemistry cannot be used to confirm the identity of AFB seen on ZN sections. Thus, while the AFB also labelled positively using immunohistochemistry, the increased time and cost necessary to produce the

immunohistochemistry sections was not offset by improved sensitivity or specificity. Consequently, it is concluded that immunohistochemistry is not the method of choice for diagnosing rabbit paratuberculosis. Recent advances in *in-situ* PCR techniques have enabled the labelling of the IS900 DNA sequence in tissues, overcoming the lack of specificity hampering the immunohistochemical technique, and allowing direct identification of *M.a. paratuberculosis* organisms in pathological sections (Sanna *et al* 2000).

3.4.5 Transmission of *M.a. paratuberculosis*

Wildlife reservoirs are thought to play a role in the epidemiology of a number of important bacterial diseases, including Lyme disease (Oliver, Jr. 1996), tularaemia and yersiniosis (Quan 1993). Possums in New Zealand and badgers in the UK have been implicated as carriers and disseminators of *M. bovis* (O'Neil and Pharo 1995, Krebs 1997), highlighting the possible effect that sylvatic cycles may have on the incidence of mycobacterial disease in ruminant livestock. The role of rabbits in the epidemiology of paratuberculosis therefore warrants close investigation.

The finding of *M.a. paratuberculosis* in the faeces of 15 rabbits highlights this as a potential transmission route from infected rabbits to domestic livestock. The number of organisms excreted in the faeces of infected rabbits was quantified by serial dilutions, and calculated to be 2.75×10^4 cfu's per gram of faeces (D. Henderson pers. comm.). The number of *M.a. paratuberculosis* organisms required to infect ruminants is unclear and likely to be variable (see section 1.4 above), however, experimental work has shown that lambs can become infected after oral inoculation with only 1×10^3 organisms, representing less than one gram of rabbit faeces. To investigate this route of disease transmission further, studies into the extent of, and grazing response of cattle to, pasture contamination with rabbit faeces were undertaken. An average of 7429 rabbit pellets were deposited per hectare per day, and, surprisingly, cattle showed no avoidance towards pasture contaminated with rabbit faecal pellets (M. J. Daniels, submitted). This is believed to be the first reported incidence of indifference by grazing cattle towards faecal contamination, contrasting with previous reports of avoidance towards pasture contaminated with either bovine or badger faeces (Forbes and Hodgson 1985, Hutchings and Harris 1997). These results indicate that ingestion of rabbit faeces containing *M.a. paratuberculosis* has the potential to be a significant source of infection for grazing ruminants, however, this hypothesis

relies upon the assumption that *M.a. paratuberculosis* "strains" from rabbits are pathogenic for cattle. This assumption is investigated in chapter 5.

3.4.6 Mycobacterium avium subsp. *paratuberculosis* in rabbit urine

The recovery of *M.a. paratuberculosis* from the urine of two rabbits is intriguing, since this is the first report of culture of *M.a. paratuberculosis* from the urine of any animal and signals a second possible transmission route of the disease from affected rabbits. It is of interest that tissues from one of the rabbits were examined histologically and classed as severely affected (Table 3-2), with extensive lesions present in the intestines and MLN, suggesting that the infection may progress to affect other body systems, possibly including the kidneys, resulting in *M.a. paratuberculosis* organisms in the urine. Further investigations into the extent of *M.a. paratuberculosis* infection in rabbits, including histopathological examination of all major body organs, including the urinary tract, are necessary to confirm this hypothesis.

3.5 Conclusion

Evidence of paratuberculosis was found in 22% of free-living rabbits from Scotland, with lesions exhibited by these rabbits similar, but not identical, to those described in ruminant paratuberculosis. Infected rabbits were found to excrete *M.a. paratuberculosis* organisms in their faeces and urine, with investigations revealing that contamination of pasture with rabbit faeces containing *M.a. paratuberculosis* and consequent ingestion of the organisms by grazing ruminants has the potential to be a significant interspecies transmission route. In response to the evidence that *M.a. paratuberculosis* can infect non-ruminant species, a wider survey of other free-living species is indicated, to define the natural host range of this organism, and clarify the extent and importance of wildlife cycles of *M.a. paratuberculosis*.

4 Wildlife Reservoirs of Paratuberculosis

4.1 Introduction

The epidemiology of paratuberculosis is not fully understood, with the role of wildlife reservoirs of the disease particularly controversial. Control and eradication plans have been proposed which rely on the absence of a significant sylvatic cycle of *M.a. paratuberculosis*, however, no large scale investigation into paratuberculosis in wildlife has been reported.

Mycobacterium avium subsp *paratuberculosis* infection has been identified in numerous free-living ruminant populations (Table 1-4), but experimental work has shown that the host range of *M.a. paratuberculosis* is not restricted to ruminant species (see section 1.11 above). These two observations have recently been linked by reports of natural paratuberculosis in wild rabbits in the Tayside region of Scotland, indicating that the organism is capable of infecting free-living, non-ruminant species (Angus 1990, Greig *et al* 1997, Greig *et al* 1999)(chapter 3).

4.2 Wildlife from Tayside

4.2.1 Hypothesis

This study investigates the natural host range of *M.a. paratuberculosis*, examining the hypothesis that the organism is capable of infecting free-living non-ruminant species, and that these species play a role in the epidemiology of paratuberculosis.

4.2.2 Materials and Methods

4.2.2.1 Collection of samples

A total of 591 samples from 18 wildlife species (Table 4-1) were collected from August 1998 to October 1999 from four farms and adjacent properties in the east of Scotland: two in Angus and two in the Perth and Kinross region (farms A, B, D and F in Table 3-1). Prior to 1994, these two regions were known as Tayside (Figure 3-1). All four farms had a history of enzootic paratuberculosis in cattle and rabbits, and one also had confirmed paratuberculosis in sheep. All

farms were greater than 20km apart from each other, and thus considered independent. Most samples were collected as part of vermin control programmes conducted by landowners, however, the hares, pheasants and badgers were road casualties while the buzzard died after colliding with electricity power lines. Post mortem examination was conducted within 12 hours of collection, with the exception of a small number of cases where a delay of up to 24 hours was unavoidable. A small number of samples (mainly stoats) were stored frozen and thus excluded from histopathological analyses.

The abdomen was the only body cavity opened at necropsy, and the following samples were taken for both culture and histopathology: duodenum, ileum, caecum, colon and MLN. A sample of liver was also included for histopathological examination. Due to the size of MLN in small mammals, collection of this tissue was not always possible. In a number of birds, the cloaca was sampled and the Bursa of Fabricius examined histologically. The caecal tonsil was used as a substitute for MLN in birds, due to the absence of aggregates of lymphoid tissue.

A sample of faeces was taken from 27 foxes, six stoats, a buzzard, 12 crows, a rook, seven rats, two wood mice, 12 sparrows, and three hares for culture. Urine was collected and cultured from 13 foxes.

4.2.2.2 Histopathology

Methods for preparation of histopathological sections are described in sections 2.1.5 and 2.1.6. Samples from one fox, 24 stoats, one badger, 12 rats, and two hares were not processed for histopathology due to advanced autolysis of the tissues. Therefore tissues from a total of 548 animals were examined histologically.

4.2.2.3 Microbiology

Methods for culturing faeces, urine and tissues are given in section 2.1.2. The method used for extraction of DNA can be found in section 2.1.3, while the detection of IS900 is detailed in 2.1.15. When attempting culture, the majority of tissues were pooled for each animal, with the exception of 17 foxes and 25 stoats, for which MLN and gut samples were cultured separately.

4.2.2.4 Statistics

Confidence intervals for the proportions were calculated using the standard method (Armitage and Berry 1994).

Table 4-1 Wildlife species collected

Common name	Scientific name	Number collected
Predators		
fox	*Vulpes vulpes*	27
stoat	*Mustela erminea*	37
weasel	*Mustela nivalis*	4
buzzard	*Buteo buteo*	1
badger	*Meles meles*	2
Scavengers		
crow	*Corvus corone*	60
rook	*Corvus frugilegus*	53
jackdaw	*Corvus monedula*	38
Prey		
rat	*Rattus norvegicus*	35
house mouse	*Mus domesticus*	89
wood mouse	*Apodemus sylvaticus*	88
field vole	*Microtus agrestis*	7
bank vole	*Clethrionomys glareolus*	19
pheasant	*Phasianus colchicus*	4
feral pigeon	*Columba livia*	59
wood pigeon	*Columba palumbus*	15
house sparrow	*Passer domesticus*	47
hare	*Lepus europaeus*	6
TOTAL		**591**

4.2.3 Results

4.2.3.1 Microbiology

Samples of tissue from a total of 591 animals were cultured and *M.a. paratuberculosis* isolated from 90, representing 10 of the 18 species examined: fox, stoat, weasel, badger, crow, rook, jackdaw, rat, wood mouse, and hare (Table 4-2). Positive faecal cultures were obtained from fox, stoat, crow, rook and wood mouse. None of the 13 samples of fox urine cultured were positive for *M.a. paratuberculosis*.

Mycobacterial species that did not contain IS900 were isolated from one wood mouse and one rat, and require further typing for identification. No pathology associated with AFB was noted in either of these animals.

4.2.3.2 Histology

Tissues from 551 animals were examined histologically and 18 samples from five species showed lesions consistent with *M.a. paratuberculosis* infection: fox, stoat, weasel, crow and wood mouse. There were similarities noted in the pathology of the carnivores (fox, stoat and weasel) in which small numbers of large macrophage-like cells were noted in the MLN and mucosa associated lymphoid tissue (MALT) of the gut (Plates **4,1-4,3**). These cells were most commonly found around the periphery of the MALT, or at the edge of a germinal centre in the cortex of the MLN. They either formed a small granuloma consisting of 10 or fewer cells, or were identified as single cells interspersed amongst the lymphocytes. A small number of AFB were identified in the cytoplasm of some of these macrophage-like cells (Plates **4,4-4,6**). As few as one AFB was identified in a section.

Acid fast bacteria were found in one crow, one wood mouse and one bank vole. The lesions noted in the crow were subtle - one section of gut contained AFB-positive cells scattered throughout the lamina propria. Only rarely were there more than five AFB seen in the cytoplasm of these cells. The liver of the crow contained numerous heterophilic granulomata which were invariably AFB negative. The lesions in the wood mouse consisted of numerous AFB-containing macrophage-like cells, both singly and in granulomata, scattered throughout the cortex of the MLN. A small number of AFB-positive cells were also noted in the villi of the small intestine (Plates **4,7-4,8**).

The liver from one bank vole contained numerous granulomata scattered throughout the parenchyma. The cytoplasm of these macrophages contained many AFB (Plates **4,9-4,11**). A small number of similar AFB-positive granulomata were noted at the base of the intestinal villi (Plates **4,12-4,13**). The AFB appeared to be more elongated than those seen in the carnivores. Similar lesions were noted in intestinal and MLN samples from a weasel.

Other pathological changes noted in the wildlife samples included intestinal worms, coccidiosis in birds and mice, and heterophilic granulomata in the liver of birds. All intestinal worms were ZN negative. Examples of incidental pathology are shown in Plates **4,14-4,17**.

Table 4-2. A summary of culture and histopathological results from wildlife collected on the four study farms.

Species	culture positive		histopathology positive	total
	tissue	faeces		
Predators				
fox[4]	23/27	3/27	12/26	24/27 (89%)
stoat	17/37	1/6	1/13	17/37 (46%)
weasel	2/4	na[2]	1/4[3]	2/4[3] (50%)
badger	1/2[1]	na	0/1	1/2 (50%)
buzzard	0/1	0/1	0/1	0/1
Scavengers				
crow	36/60	4/12	1/60	36/60 (60%)
rook	3/53	1/1	0/53	3/53 (6%)
jackdaw	1/38	na	0/38	1/38 (3%)
Prey				
rat	3/35	0/7	0/23	3/35 (9%)
house mouse	0/89	na	0/89	0/89
wood mouse	3/88	2/2	1/88	3/88 (3%)
field vole	0/7	na	0/7	0/7
bank vole	0/19	na	0/19[3]	0/19[3]
pheasant	0/4	na	0/4	0/4
feral pigeon	0/59	na	0/59	0/59
wood pigeon	0/15	na	0/15	0/15
house sparrow	0/47	0/12	0/47	0/47
hare	1/6	0/3	0/4	1/6 (17%)
TOTAL	90/591	11/71	18/551	91/591 (15%)

[1]One badger was collected from outside the sample area. This animal was positive on culture for *M.a. paratuberculosis*, but no lesions were noted on histopathological examination.
[2]na: procedure not carried out on samples
[3]AFB were noted from one bank vole and one weasel, but were judged unlikely to be *M.a. paratuberculosis* and not recorded as positive samples in this table.
[4] Species in bold had evidence of *M.a. paratuberculosis* infection

4.2.3.3 Statistics

The prevalence of *M.a. paratuberculosis* infection is depicted in Figures 4-1 and 4-2. Figure 4-1, based on the results of culturing the tissues, shows that the species fall into four groups - group 1 have a high, well defined prevalence of *M.a. paratuberculosis* infection (fox, stoat and crow), while group 2 have infection present but at a low rate (rook, jackdaw, rat, and wood mouse). The hare, weasel and badger make up group 3, with *M.a. paratuberculosis* infection detected, but imprecise data due to the low number of animals sampled, and group 4 includes the pheasant, buzzard, field vole, bank vole, house mouse, feral pigeon, wood pigeon, and sparrow. *Mycobacterium avium* subsp. *paratuberculosis* was not detected in any of these species. The histopathological data is presented in Figure 4-2, and indicates a correlation with Figure 4-1, but also the lower sensitivity of this technique when compared to the culture of tissues.

Figure 4-1 The prevalence and 95% confidence intervals of *M.a. paratuberculosis* infection in wildlife based on results of culture from tissues of wildlife

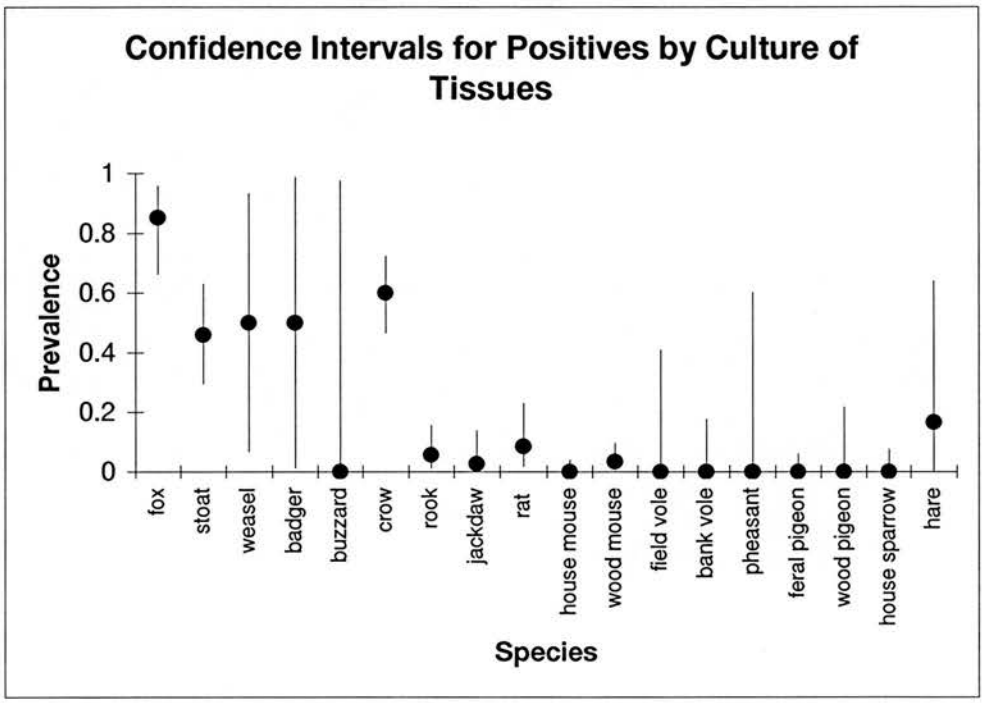
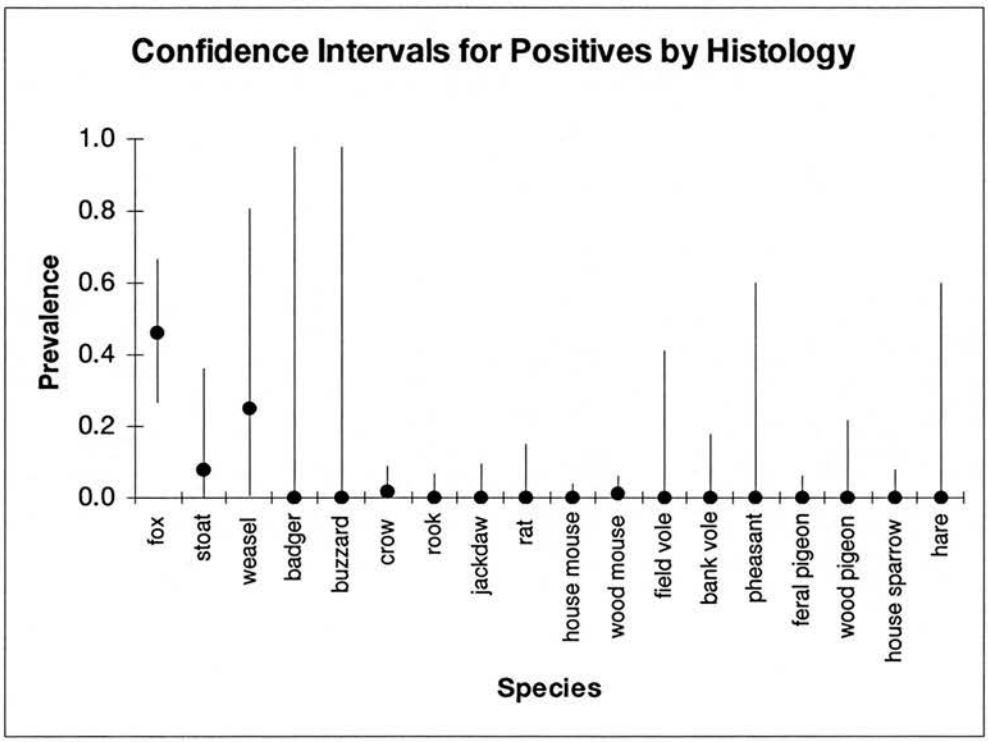


Figure 4-2 The prevalence and 95% confidence intervals of *M.a. paratuberculosis* infection in wildlife based on the results of histopathological analysis of tissues



4.3 Urban foxes

4.3.1 Hypothesis

Finding foxes infected with *M.a. paratuberculosis* on farms with a history of enzootic paratuberculosis in domestic ruminants (see section 4.2 above) suggests that the two cycles may be linked. This would imply that a group of foxes from an urban environment, with no known exposure to *M.a. paratuberculosis,* would have no evidence of paratuberculosis. To investigate this hypothesis, 27 foxes from London were collected and examined.

4.3.2 Materials and Methods

Twenty seven urban foxes were collected from the Croyden and Dartford areas of London. All foxes were casualties brought into wildlife rescue centres. A note was made of the cause of death (where it could be ascertained), the sex, and the age of the animal (Table 4-3). A sample of MLN and terminal ileum was collected from each animal for culture. Samples for histopathology were collected from three of the 27 foxes.

4.3.2.1 Microbiology

Samples for culture were stored at –20^{o}C for between one and six months before being cultured. Samples of MLN and terminal ileum were cultured separately using the methods described in 2.1.2.

4.3.2.2 Histopathology

Samples were fixed in formal saline for a minimum of 24 hours, before processing and preparation of sections as described in sections 2.1.5 and 2.1.6.

4.3.3 Results

4.3.3.1 Microbiology

No *M.a. paratuberculosis* organisms were isolated from any tissue (Table 4-3).

4.3.3.2 Histopathology

No evidence of paratuberculosis infection was seen in any of the three samples examined.

Table 4-3. Details of the 27 foxes and results of the microbiological examination of the terminal ileum and MLN from each animal.

sex	age	clinical signs	culture of tissues	
			terminal ileum	MLN
M	juvenile	spinal trauma	-	-
F	juvenile	RTA	-	-
F	juvenile	RTA	-	-
F	juvenile	CNS disorder	-	-
M	young adult	mange	-	-
M	young adult	collapsed	-	-
F	young adult	CNS disorder	-	-
F	young adult	jaundice	-	-
M	adult	maggots	-	-
M	adult	maggots	-	-
M	adult	infected wounds	-	-
M	adult	gangrene	-	-
M	adult	collapsed	-	-
M	adult	collapsed	-	-
M	adult	CNS disorder	-	-
M	adult	RTA	-	-
M	adult	RTA	-	-
M	adult	broken back	-	-
M	adult	trapped	-	-
M	adult	jaundice	-	-
F	adult	CNS signs	-	-
F	adult	CNS disorder	-	-
F	adult	RTA	-	-
F	adult	jaundice	-	-
F	adult	mange	-	-
F	adult	fight wounds	-	-
F	old adult	abscess	-	-

RTA: road traffic accident
CNS: central nervous system
M = male
F = female

4.4 Northern England

4.4.1 Hypothesis

Surveys in Tayside have revealed *M.a. paratuberculosis* infection in rabbits and other wildlife species from paratuberculosis-affected farms (Greig *et al* 1999)(chapter 3, and section 4.2). To determine the geographical extent of *M.a. paratuberculosis* infection of non-ruminant wildlife, a pilot study was undertaken, screening wildlife on paratuberculosis-affected farms in Northern England.

4.4.2 Materials and methods

Four farms in Northern England were included in the study. Two (farms 1 and 3) had a history of paratuberculosis in domestic cattle on the farm, one had a history of paratuberculosis in sheep (farm 2), while the fourth farm (farm 4) was a large estate with anecdotal evidence of mycobacterial infection in free-living deer. Farms 1 and 3 were in North Yorkshire, farm 2 in Cumbria, and farm 4 in County Durham. All farms were at least 16 kilometres distant from each other.

All carcasses were collected and a post mortem carried out within 24 hours. Carcasses were sexed, the abdomen was opened, and a note made as to whether the animal was pregnant or lactating. Samples of duodenum, jejunum, terminal ileum, ileocaecal valve, descending colon, MLN and liver were collected from all animals and fixed in 10% buffered formal saline for histopathological analysis. A separate sample of terminal ileum and MLN from each animal was pooled in a sterile container for storage and possible future culture. Fetal samples from two foxes and one rabbit were also included for histopathological examination.

4.4.2.1 Histopathology

Sections were prepared for histopathological analysis and stained using the H&E and ZN method as described in sections 2.1.5 and 2.1.6.

4.4.2.2 Microbiology

Tissue samples from rabbits, hares and deer were stored at -70°C for future culture, pending histopathology results. Tissues from foxes were cultured as described earlier (2.1.2).

4.4.3 Results

Fifty four animals (3 roe deer, 3 foxes, 4 hares, and 44 rabbits) were collected from the four study farms (Table 4-4).

Table 4-4 Details of the number, sex, and reproductive status of animals collected from each farm.

	Deer			Foxes		Hares		Rabbits	
	M	F	?	M	F	M	F	M	F
Farm 1	0	1 (1)[1]	2	1	2 (2)	2	1	2	8 (1) [3][2]
Farm 2	0	0	0	0	0	1	0	3	7 (0) [4]
Farm 3	0	0	0	0	0	0	0	4	8 (3) [3]
Farm 4	0	0	0	0	0	0	0	7	5 (0) [3]
Total	0	1 (1)	2	1	2 (2)	3	1	16	28 (4) [13]
	3			3		4		44	

[1] ( ) = number of females pregnant
[2] [ ] = number of females lactating

4.4.3.1 Pathology

On histopathological examination, no AFB were identified in the tissues of any animal. No pathology consistent with that seen in a case of paratuberculosis was found in any of the tissues. Incidental pathology noted included coccidial oocysts in the intestinal epithelium and intestinal worms (Table 4-5). In addition, two rabbits showed evidence of hepatic coccidiosis.

4.4.3.2 Microbiology

No *M.a. paratuberculosis* cultures were recovered from the tissues of the foxes. In the light of the negative histopathology results from the other species, no other tissues were cultured.

4.4.3.3 Statistics

Foxes: 0/3 foxes were positive for the presence of *M.a. paratuberculosis*. The 95% confidence interval for the prevalence of *M.a. paratuberculosis* infection in foxes is therefore (0, 71%). The prevalence of the disease in foxes in Northern England is significantly less than the 89% infection rate reported in Tayside (p=0.003, where this value is the probability of observing a number of positives at least as extreme as 0/3, given that the prevalence of infection is 89%)

Rabbits: 0/44 rabbits were positive for the presence of *M.a. paratuberculosis*. The 95% confidence interval for the prevalence of *M.a. paratuberculosis* infection in rabbits is calculated as (0, 8%). The prevalence of the disease in rabbits in Northern England is significantly less than the 22% infection reported in Tayside ($p<0.001$ where this value is the probability of observing a number of positives at least as extreme as 0/44, given that the prevalence of infection is 22%).

Table 4-5 Histological evidence of intestinal worms and coccidiosis in each species

	Fox	Rabbit	Hare	Deer
Intestinal coccidiosis	0	12	3	0
Intestinal worms	1	16	2	0
Total examined	3	44	4	3

4.5 Discussion

Study 4.2 revealed evidence of natural *M.a. paratuberculosis* infection in a wide range of wildlife species from paratuberculosis-affected farms in east Scotland. *M.a. paratuberculosis* was isolated from tissues of the fox, stoat, weasel, badger, crow, rook, jackdaw, rat, wood mouse, and hare, and histopathological lesions consistent with *M.a. paratuberculosis* infection were noted in tissues from the fox, stoat, weasel, crow and wood mouse. This survey is the first to report natural infection with *M.a. paratuberculosis* in these animals, and extends the earlier reports of paratuberculosis in rabbits (Angus 1990, Greig *et al* 1997, Greig *et al* 1999) (chapter 3). The infection of non-ruminant free-living species with *M.a. paratuberculosis* appears to be concentrated in the carnivorous and scavenging species such as fox, stoat, weasel, crow, rook and jackdaw, suggesting that predation is the major route of transmission in sylvatic cycles of the organism, rather than via the faeco-oral route, as is the case in ruminant paratuberculosis.

4.5.1 Paratuberculosis in carnivores and carrion eating birds

The Tayside investigation provides strong evidence of enzootic natural *M.a. paratuberculosis* infection of foxes. Of those examined, 89% showed evidence of infection. Histopathological lesions were consistent with infection by a slow growing mycobacterial species, such as *M.a. paratuberculosis,* and there was a correlation between culture of tissues and faeces, and histopathological lesions in each animal (Table 4-6).

Table 4-6 The results from analysis of samples from foxes, revealing the relationship between culture of *M.a. paratuberculosis* and histopathological lesions.

	total	*presence of lesions in tissues*	*faecal culture positive*
Tissue culture positive*	23	11/22	3/23
Tissue culture negative	4	1/4	0/4
Total	27	12/26	3/27

*comprised of animals positive by culture of intestine, MLN, or both.

Reports of mycobacterial infection in free-living canid species are rare. When *M. bovis* was cultured from the lymph nodes of three free-living coyotes (*Canis latrans*) in Michigan (BruningFann *et al* 1998), the authors hypothesised that these animals were infected by ingesting

tuberculous deer. Interestingly, in the same survey, *M. bovis* was not recovered from five foxes and two badgers, however, the organism has been cultured from the tissues of 1.15% of 954 foxes examined in the UK (Krebs 1997). There are a small number of reports in the literature of domestic dogs suffering from slow growing mycobacterial infections, including *M. avium* (Shackelford and Reed 1989) and *M. avium intracellulare* (Miller *et al* 1995). Evidence from Australia suggests that dogs affected with canine leproid granuloma syndrome are suffering from infection with a novel, slow growing mycobacterial species (Malik *et al* 1998, Hughes *et al* 2000).

Study 4.2 also revealed for the first time natural *M.a. paratuberculosis* infection of the weasel and stoat. There was a significant discrepancy between the results of culture and histopathology of the latter, with lesions noted in only one stoat, while 17 were positive by culture, however, 24 samples showed advanced autolysis, which precluded meaningful histopathological examination. It is noteworthy that natural *M. bovis* infection has been reported in feral ferrets (*Mustela furo*) (Lugton *et al* 1997) and stoats (Cooke *et al* 1993), as well as *M. microti* and *M. genavense* infections in pet ferrets (van Soolingen *et al* 1998, Lucas *et al* 2000).

The isolation of *M.a. paratuberculosis* from the lymph node of the stoat and fox rules out the possibility that the positive tissue cultures were due to recently ingested infected tissue in the intestinal lumen, and indicates that these animals are chronically infected with *M.a. paratuberculosis*. It also suggests that the pathogenesis of paratuberculosis in carnivores is similar to the early, subclinical infections described in ruminants (Perez *et al* 1996), with the organism undergoing a long incubation period in macrophages in lymphoid tissue. The presence in both fox and stoat of AFB in macrophage-like cells, as well as the more frequent isolation of *M.a. paratuberculosis* from MLN, compared to intestinal tissue, support this suggestion (Table 4-7, Table 4-8). However, while early *M.a. paratuberculosis* infection in carnivores, rabbits and ruminants appears to be similar, there is no evidence to show that free-living carnivorous species eventually develop extensive granulomatous lesions analogous to those reported in ruminants and rabbits (see section 1.6 and chapter 3). The carnivores may be able to perpetually control the multiplication of *M.a. paratuberculosis*, and limit infection to small areas in the lymphoid compartments of the intestine.

Table 4-7 A comparison of MLN and intestinal culture in a subset of 16 foxes

	intestine +	*intestine -*	*Total*
MLN +	9	3	12
MLN -	1	3	4
Total	10	6	

Table 4-8 A comparison of MLN and intestinal culture in a subset of 20 stoats

	intestine +	*intestine -*	*Total*
MLN +	6	4	10
MLN -	1	9	10
Total	7	13	

Mycobacterium avium subsp *paratuberculosis* was isolated from tissues of 36 crows, three rooks and one jackdaw, although AFB were noted in tissue sections from just one crow (Table 4-2). The difference between culture and histopathological results may be due to the subtlety of the *M.a. paratuberculosis* lesions in these species, or could indicate passive transmission of ingested *M.a. paratuberculosis*-infected material through the gastrointestinal system of these species. While *M.a. paratuberculosis* infection of free-living avian species has not been reported previously, they are known to be susceptible to infection with slow growing mycobacterial species (Thoen *et al* 1977, Saxegaard 1981, Saxegaard 1985, Smit *et al* 1987, Morishita *et al* 1998).

The natural diets of foxes, stoats and weasels consists predominately of rabbits and small rodents (King 1991, Leckie *et al* 1998), while the *M.a. paratuberculosis*-positive birds in this survey (crows, rooks and jackdaws) are carrion eaters (Mason and Macdonald 1995), and known to ingest rabbit tissue (Waite 1985), suggesting that the infected rabbit population on the farms was the most likely source of *M.a. paratuberculosis* for the carnivores, rather than infected ruminants or contaminated environment. Thus, it is probable that *M.a. paratuberculosis* infection of carnivores relies upon the sylvatic cycle of infection in the rabbits. Other examples of infectious agents transmitted from rabbits to carnivores via predation include rabbit haemorrhagic virus (Leighton *et al* 1995) and the intestinal parasite *Taenia pisiformis* (Richards *et al* 1995).

4.5.2 Paratuberculosis in rodents and badgers

Mycobacterium avium subsp *paratuberculosis* was cultured from the tissues of three rats and three wood mice, with AFB noted in the MLN and intestine of one of the three wood mice. The source of the *M.a. paratuberculosis* infection in these animals is not clear, but scavenging on the floors of barns in which cattle are housed is one possibility. While experimental infection of *M.a. paratuberculosis* in rodents has been reported (Francis 1943, Larsen and Miller 1979), this is the first evidence of natural infection in rats or mice.

Two badgers were examined in this survey, but only one was collected from within the sample area. The tissues from this animal were too autolytic for histopathological examination, and no *M.a. paratuberculosis* organisms were grown from them. The second badger was taken from an area approximately 120 kilometres north east of the study area, and within 10 kilometres of a farm known to have paratuberculosis-affected ruminant livestock. *Mycobacterium avium* subsp *paratuberculosis* was grown from the tissues of this animal, but no lesions consistent with a diagnosis of paratuberculosis were noted from histopathological examination. Badgers in the south west of the UK develop natural *M. bovis* infection, and may play a crucial role in the epidemiology of bovine tuberculosis (Krebs 1997, Hutchings and Harris 1999), therefore natural infection with another species of mycobacterium is possible. Badgers are known to eat mammals including rabbits (Kruuk and Parish 1982, Neal and Cheeseman 1991), implying that, similar to the other carnivores discussed above, infected rabbits may be a source of *M.a. paratuberculosis* for this species.

4.5.3 Other wildlife species

As the ZN staining method is not specific for *M.a. paratuberculosis*, merely identifying AFB in the tissues of animals in this survey is not conclusive proof of *M.a. paratuberculosis* infection. Large numbers of AFB were noted in the liver, intestines and lymph node of a bank vole, and the lymph node and intestines of a weasel, but no *M.a. paratuberculosis* organisms were cultured from the tissues of either animal. The particularly extensive liver lesions of the bank vole and very high numbers of AFB in the MLN of the weasel were not consistent with cases of paratuberculosis, therefore, when considered with the negative culture result, it is probable that the AFB seen were another acid fast species, possibly *M. microti*, the causative agent of vole tuberculosis (Wells 1937, Brooke 1941, van Soolingen *et al* 1998).

No evidence was found of *M.a. paratuberculosis* infection of house mice, feral pigeons, wood pigeons, house sparrows, field voles, pheasants or a buzzard. As only small numbers of pheasants, hares, field voles and buzzards were examined, it is not possible to conclude what role these species might play in the epidemiology of ruminant paratuberculosis.

4.5.4 Interspecies transmission routes

The occurrence of *M.a. paratuberculosis* in a wide range of free-living species is of greater significance if the organism can be passed from these animals to farmed livestock, resulting in an increased prevalence of ruminant paratuberculosis. The discovery of *M.a. paratuberculosis* in the faeces of a number of wildlife species suggests that the greatest risk of transmission to domestic ruminants would come from the faecal contamination of food and water. *Mycobacterium avium* subsp *paratuberculosis* is capable of surviving for long periods of time in the environment (see section 1.2.1 above), and all species which were found to be infected are known to contaminate either stored feed (rodents and birds) or pasture (carnivores), which could then be ingested by domestic ruminants. Experimental work has revealed that, while cattle and sheep show avoidance to concentrate feed contaminated with either rat or mouse faeces, this avoidance is not sufficient to prevent ingestion of significant numbers of faecal pellets. Monitoring of stored feed on the four farms in the survey revealed 66 rodent and 22.3 bird faecal pellets were deposited per m^2 of feed per month, representing a significant potential route for disease transmission to livestock (M. J. Daniels submitted). Other diseases resulting from farmed animals ingesting food or water contaminated by wildlife include toxoplasmosis (Buxton 2000), cryptosporidiosis (Chalmers *et al* 1997), leptospirosis (Webster and Macdonald 1995), salmonellosis and campylobacter infection (Linklater 2000).

4.5.5 Study of urban foxes and some wildlife species from Northern England

In contrast to the results from Tayside (see section 4.2), the smaller study in Northern England (described in section 4.4) did not reveal evidence of *M.a. paratuberculosis* infection in wildlife from four paratuberculosis-affected farms. It is statistically unlikely that these farms have either an infected fox or rabbit population at levels comparable to those found in Tayside.

Similar results were gained when urban foxes were examined in section 4.3. No evidence of *M.a. paratuberculosis* infection was noted in any of the foxes collected from the London area.

4.5.6 Conclusion

The natural host range of *M.a. paratuberculosis* was found to include the fox, stoat, weasel, badger, crow, rook, jackdaw, rat, wood mouse and hare, indicating that non-ruminant wildlife infection is widespread. The concentration of *M.a. paratuberculosis* infection in carnivorous and scavenging free-living species suggests that predation, most probably of infected rabbits, is the most common means of transmission of the organism. Use of molecular typing techniques to compare the isolates of *M.a. paratuberculosis* from the species examined in this study with those found in rabbits and domestic ruminants may help confirm this hypothesis, and add to our knowledge of the epidemiology of *M.a. paratuberculosis*.

The results from the study recorded in section 4.3 infer that paratuberculosis in foxes is associated with paratuberculosis in domestic livestock, possibly via *M.a. paratuberculosis* infection of rabbits. However, the lack of evidence of *M.a. paratuberculosis* infection in both foxes and rabbits from paratuberculosis-affected farms in Northern England (section 4.4) revealed that the mere presence of paratuberculosis in domestic ruminants is insufficient for infection to pass to either the rabbit or fox population. Further studies are required to determine the factors which influence the initiation and consequent prevalence of *M.a. paratuberculosis* infection of wildlife.

5 Experimental Studies of Interspecies Transmission of *M.a. paratuberculosis*

5.1 Hypothesis

Natural *M.a. paratuberculosis* infection has recently been discovered in free-living rabbits and other wildlife species (Greig *et al* 1997, Greig *et al* 1999) (chapter 3, chapter 4), suggesting that the epidemiology of paratuberculosis may involve non-ruminant wildlife cycles of the organism. A wildlife reservoir of *M.a. paratuberculosis* could have a significant effect on the disease in domestic livestock, acting as a continual source of infection, as well as promoting farm to farm spread of the organism. However, the extent to which the cycle of *M.a. paratuberculosis* in wildlife and domestic livestock are linked is unclear. It is possible that, for example, rabbits and cattle are infected with species-specific strains of *M.a. paratuberculosis*, similar to the situation described in sheep and cattle in Australia (see section 1.9 above), resulting in mutually exclusive cycles of the organism, with little, if any, interspecies transmission of disease.

To investigate the possibility of transmission of *M.a. paratuberculosis* between rabbits and cattle, two parallel experiments were carried out. An isolate of *M.a. paratuberculosis* from a naturally infected, free-living rabbit and an isolate from a clinically affected bovid were inoculated orally into young calves (section 5.2) and neonatal rabbits (section 5.3), and the resulting pathological changes investigated and compared.

5.2 Calf transmission experiment

5.2.1 Materials and Methods

Eighteen newborn male Holstein - Friesian cross calves were allocated to one of three groups: group Co_b (six calves) was designated the control group and treated with PBS, eight calves were placed in group Ra_b ("rabbit group") and inoculated with the leporine isolate (R7) of *M.a. paratuberculosis*, while the remaining four calves were allocated to group Bo_b ("bovine group"), and inoculated with a bovine isolate (F13) of *M.a. paratuberculosis*. Each group of calves was housed separately, with animals individually penned until six weeks old.

Each week for three weeks, calves in groups Ra_b and Bo_b were given an oral solution of *M.a. paratuberculosis* suspended in 5ml PBS, while calves in group Co_b received only PBS. The first dose was administered within five days of birth. Preparation of the inoculum is described in section 2.1.1. Retrospective counts (see section 2.1.4 above) were carried out on the inoculum to obtain a more accurate estimation of the dose received by the calves. Details of group numbers and doses are summarised in Table 5-1.

Table 5-1 Experimental design, showing the number of animals in each of the three groups, and the inoculation doses.

	Inoculum	Number in group	Average dose	Total dose
Group Co_b	Control (PBS)	6	0 cfu	0 cfu
Group Ra_b	Rabbit isolate	8	$3.2x10^8$ cfu per dose	$9.6x10^8$ cfu
Group Bo_b	Bovine isolate	4	$1.85x10^8$ cfu per dose	$5.5x10^8$ cfu

Faecal samples were taken at the time of first inoculation, weekly for the first eight weeks, and thereafter monthly until the calves were six month old, when they were killed by an intravenous injection of barbiturate and a post mortem examination carried out.

Representative samples of tissues were taken from each animal during the post mortem examination for histopathological and microbiological analysis (Table 5-2). Details of methods for the preparation of histological sections are described in sections 2.1.5 and 2.1.6. The technique used for culture of tissue and faeces, with confirmatory IS900 PCR, can be found in sections 2.1.2, 2.1.3 and 2.1.15.

Table 5-2 Summary of the samples taken from the calves during post mortem examination for histopathological and microbiological examination

	duodenum	jejunum	JPP	IPP	terminal ileum	proximal MLN	distal MLN	ICV	ICLN	liver	retropharyngeal lymph node	submandibular lymph node
immediate culture			✔				✔					
future culture[1]				✔	✔	✔		✔				
histopathology	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔

[1]Samples for future culture were stored at -70^0C
JPP = jejunal Peyer's patch, IPP = ileal Peyer's patch, MLN = mesenteric lymph node, ICV = ileocaecal valve, ICLN = ileocaecal lymph node.

5.2.2 Results

Four animals required veterinary intervention while on the study, details of which are given in section 5.5 below. No animal exhibited signs of diarrhoea or weight loss during the six month incubation period.

5.2.2.1 Microbiology

Culture of faeces during experiment

A total of 229 faecal samples were cultured from the calves during the experiment. *Mycobacterium avium* subsp. *paratuberculosis* was isolated from six samples, originating from five calves, all of whom were in group Ra_b (Table 5-4). The positive faecal samples were produced at time points throughout the experiment, from week one to week 27.

Culture of tissues collected during post mortem examination

Mesenteric lymph node and JPP were cultured from all calves, and *M.a. paratuberculosis* isolated from seven out of eight animals in group Ra_b and three out of four animals in group Bo_b. No bacterial isolates were recovered from any animal in group Co_b. Bacteriological culture results from each group are summarised in Table 5-3, and results from each inoculated calf in Table 5-4.

5.2.2.2 *Pathology*

No abnormalities were noted upon gross examination of any animal, however, three out of eight animals in group Ra_b and two out of four animals in group Bo_b developed histopathological lesions consistent with a diagnosis of early *M.a. paratuberculosis* infection (Table 5-3). No lesions indicative of *M.a. paratuberculosis* infection were noted in any tissue from animals in group Co_b (the control group). The pathological changes identified in groups Ra_b and Bo_b are summarised in Table 5-5, and consisted of circumscribed, non-encapsulated granulomata containing macrophages, epithelioid cells, and occasional giant cells. A variable number of AFB were present in the cells of the granulomata - no AFB were visible in some granulomata. The most common site for lesions to be noted was the lamina propria and underlying lymphoid tissue of the ICV (Plates **5,1-5,2**), and its draining lymph node, the ICLN (Plates **5,3-5,5**). However, lesions were noted in the jejunum of one animal in group Bo_b, where granulomata were identified in both the JPP and the overlying lamina propria, with a small number of AFB present in the granulomata of the JPP (Plate **5,6**).

Pathology consistent with chronic parasitism was evident in animals from all three groups, with the most likely cause of these lesions considered to be intestinal coccidiosis. The changes noted included pyogranulomata containing mainly polymorphonuclear cells in the MALT (Plate **5,7**), and coccidial oocysts in association with epithelial cells of the intestine (Plate **5,8**). Based on the severity of the changes, each animal was given a score from 0-3. Animals with no evidence of parasitic infection were given a score of 0, and if only very mild evidence of coccidial infection was present (fewer than five oocysts), the animal was given a grade of 1. If greater than five oocysts were noted, the animal was given a score of 2, while grade 3 represented more severe chronic changes, including pyogranulomata, encapsulated abscesses (Plate **5,9**), and varying numbers of coccidial oocysts. The scores were averaged for the whole group (Table 5-3) to allow a comparison of the severity of intestinal coccidiosis infection between groups to be made.

5.2.2.3 *Statistics*

Using Fisher's exact test, a statistically significant difference ($p<0.05$) was found between the culture results of group Co_b and group Ra_b, as well as between group Co_b and group Bo_b. The corresponding statistical differences between the histopathology results were close to, but not below the 5% mark (Table 5-3). No significant differences were detected either by culture or histopathology between the two treated groups – Ra_b and Bo_b.

Table 5-3 A summary of group microbiological and histopathological results for tissues collected at postmortem*. A comparison of the average parasitism score is also shown (see page 102).

	Culture			Histopathology	Average parasitism score
	JPP	MLN	JPP or MLN		
Group Co_b	0/6	0/6	0/6	0/6	1.3
Group Ra_b	2/8	5/8	7/8[1]	3/8[3]	1.375
Group Bo_b	3/4	3/4	3/4[2]	2/4[4]	1.25

*results are expressed as the number of positive animals out of the total number in the group
Significant differences between each infected group and the control group were determined using Fisher's exact test. Probability values are: [1]p=0.002,[2]p=0.02, [3]p=0.13, [4]p=0.07

Table 5-4 A comparison of the microbiological and histopathological results of each calf from groups Ra_b and Bo_b.

Group	Calf id	Age at first inoculation (days)	Culture of faeces	Culture of tissues	Histopathology
Ra_b	1504	4	+ (27)[2]	-	-
Ra_b	1510	1	-	+	-
Ra_b	1511	1	+ (12)	+	-
Ra_b	1514	3	-	+	-
Ra_b	1522[1]	2	-	+	+
Ra_b	1523	2	+ (4)	+	+
Ra_b	1525	2	+ (22)	+	+
Ra_b	1526	3	+ (1 and 2)	+	-
Bo_b	1540	3	-	-	-
Bo_b	1543[1]	2	-	+	+
Bo_b	1545	2	-	+	+
Bo_b	1547[1]	5	-	+	-

[1]animal received antibiotic therapy while on trial (see Table 5-5).
[2]number in parentheses is the week of the experiment in which the positive faecal sample was collected.

Table 5-5 A summary of the pathological changes noted in the tissues examined from three calves in group Ra_b and two calves in group Bo_b, six months after inoculation with *M.a. paratuberculosis* organisms.

Animal id	Group	jejunum				ICV				ICLN	
		villi		ly tissue		villi		ly tissue			
		path	AFB	path	AFB	path	AFB	path	AFB	path	AFB
1522	Ra_b	-	-	-	-	++	++	-	-	-	-
1523	Ra_b	-	-	-	-	-	-	-	-	-	+
1525	Ra_b	-	-	-	-	++	+	+	-	+	+
1543	Bo_b	-	-	-	-	-	-	+	-	+++	+
1545	Bo_b	++	-	++	+	++	+	+	-	+++	++

ly = lymphoid, path = extent of pathological changes, AFB = acid fast bacilli, ICV = ileocaecal valve, ICLN = ileocaecal lymph node

Scoring system:

path : + 1-2 small granulomata
++ Between 2-10 granulomata
+++ Greater than 10 granulomata

AFB: + 1-5 clumps of bacteria identified
++ moderate numbers of AFB present
+++ abundant numbers of AFB present

5.2.3 Discussion

This experiment has shown that an isolate of *M.a. paratuberculosis* from a naturally infected rabbit is capable of infecting young calves and causing lesions consistent with a diagnosis of early paratuberculosis. This supports the hypothesis that transmission of paratuberculosis from rabbits to cattle can occur.

5.2.3.1 Histopathological lesions

The pathological changes identified in animals inoculated with both the rabbit and bovine isolate are consistent with early lesions of paratuberculosis described by other workers (Angus and Gilmour 1971, Juste *et al* 1994) (summarised in section 1.7.1.2). Tissues from all animals with histopathological changes indicative of early paratuberculosis yielded isolates of *M.a. paratuberculosis* when cultured, thus confirming the identity of the AFB noted in the tissue sections.

In chapter 6, a lamb experimental model of paratuberculosis is described. After a four week incubation period, lesions noted in the inoculated lambs were small, confined to the JPP, and contained no visible AFB. In contrast, the lesions noted in the calves in this experiment suggested the presence of more advanced disease, with pathological changes noted in the JPP,

ICV and ICLN. The lesions were more extensive when compared to those in chapter 6, and the majority contained moderate to numerous numbers of intracellular AFB. The finding of lesions in the ICV and ICLN is significant, since the terminal ileum and ICV are reported as the most common sites of paratuberculosis lesions in clinical cases (see section 1.6 above), suggesting that the infection in the calves was developing into classical end-stage paratuberculosis.

5.2.3.2 Prevalence of infection in groups Ra_b and Bo_b

There was a high prevalence of infection in the calves in groups Ra_b and Bo_b - all except two out of a total of 12 calves inoculated with *M.a. paratuberculosis* had evidence of infection at post mortem. This high prevalence of infection may reflect the housing of the calves, since calves in each group were in contact with each other, permitting transmission of organisms from one animal to another. It may also reflect the suspected increased susceptibility of young animals to infection (see section 1.4 above), as all calves in this study were exposed to *M.a. paratuberculosis* in the first week of life (Table 5-4).

5.2.3.3 Effect of antimicrobial therapy

Three animals were treated with antimicrobial therapy while on the trial (described in section 5.5 below), and all three were found to be infected with *M.a. paratuberculosis* at post mortem (Table 5-4), thus underlining the inability of these drugs to gain access to the intracellular *M.a. paratuberculosis* organism and kill it. However, this study did not aim to assess the effect of antimicrobial treatment on *M.a. paratuberculosis* infection, and, apart from the observation that, in this study, they did not appear to prevent long term infection, no further statement can be made about the effectiveness of antimicrobials against *M.a. paratuberculosis* infection.

5.2.3.4 Faecal shedding of organisms

Very few faecal samples from the calves yielded *M.a. paratuberculosis* on culture. From a total of 229 samples, only six yielded *M.a. paratuberculosis* organisms when cultured (Table 5-4). These faecal culture results underline the intermittent nature of the shedding of *M.a. paratuberculosis* organisms by subclinically infected animals, which has been reported by other workers (Chaitaweesub *et al* 1999, McDonald *et al* 1999).

The results from faecal cultures do not correlate well with the results from the post mortem analysis of samples. Animal 1504 produced a faecal sample containing *M.a. paratuberculosis* in

week 27, but showed no evidence of *M.a. paratuberculosis* infection in either the histopathological analysis or culture of tissues collected at post mortem. Conversely, calves 1522, 1543 and 1545 had lesions with moderate to abundant numbers of AFB present in the intestines, and *M.a. paratuberculosis* was recovered from the MLN and JPP of all three calves. However, no organisms were recovered from faecal samples of these animals during the six months of the experiment. The intermittent nature of faecal shedding of *M.a. paratuberculosis* organisms from infected animals as well as the poor correlation between severity of infection and faecal culture highlights the difficulty in using this technique to diagnose subclinically infected animals.

Mycobacterium avium subsp *paratuberculosis* was isolated from one faecal sample taken at the time of first inoculation (animal 1526, week 1). This is most likely due to contamination of the sample or cross infection, and is thought to be unlikely to indicate prior infection with *M.a. paratuberculosis*. The farm the calves were sourced from is monitored regularly for paratuberculosis, and the six control calves included in the experiment (group Co_b) showed no evidence of *M.a. paratuberculosis* infection.

5.2.3.5 Comparison of results from group Ra_b and Bo_b

This experiment was designed to compare the pathogenicity of a cattle and a rabbit isolate of *M.a. paratuberculosis* when inoculated into young calves. The results from the culture of tissues are comparable in both groups – samples from seven out of eight calves in group Ra_b and three out of four calves in group Bo_b yielded isolates of *M.a. paratuberculosis*. A similar proportion of animals in each group developed lesions – three out of eight in group Ra_b, and two out of four in group Bo_b. Consequently, no statistical differences were detected between the two treated groups. However, some disparaties between the groups were evident. The lesions noted in the two animals from group Bo_b were more extensive with a higher number of AFB present when compared to group Ra_b, and while no animals from group Bo_b were ever detected shedding *M.a. paratuberculosis* in their faeces, five out of eight calves in group Ra_b produced a faecal sample containing *M.a. paratuberculosis*. This could indicate that, while the bovine isolate may produce more extensive lesions, the rabbit isolate has the potential to be more easily transmitted between animals. Further experiments are required to investigate these observations and determine if they do represent the characteristics of different *M.a. paratuberculosis* strains.

5.3 Rabbit transmission experiment

5.3.1 Materials and Methods

Five litters of newborn New Zealand white rabbits, containing a total of 25 kits, were allocated to one of three groups. Group Co_r, made up of two litters with a total of nine kits, was designated the control group, and received 0.25ml of sterile PBS orally at each inoculation. Group Bo_r contained one litter with eight kits, and was inoculated with a bovine isolate (F13) of *M.a. paratuberculosis*, while group Ra_r consisted of two litters, a total of eight kits, which were inoculated with a leporine isolate (R7) of *M.a. paratuberculosis*. The inoculum was prepared as described earlier (in section 2.1.1). Each kit was inoculated orally on the day of birth (day 0), and then every other day until day nine, a total of six inoculations altogether. The experimental details are summarised in Table 5-6. Retrospective counts (described in section 2.1.4) of inocula were performed to allow an accurate estimation of the total dose received by the kits (Table 5-6).

Table 5-6 An outline of the experiment designed to compare the virulence of a bovine and a leporine isolate of *M.a. paratuberculosis* by oral inoculation of young rabbits, detailing the group sizes and inoculation doses.

	Number in group	Inoculum	Total number of organisms	Number necropsied at six months
Dams	5 in total	none	0	0[a]
Group Co_r	9 kits (2 litters)	PBS	0	4
Group Bo_r	8 kits (1 litter)	*M.a. paratuberculosis* (bovine isolate)	$5.02x10^7$cfu	1[b]
Group Ra_r	8 kits (2 litters)	*M.a. paratuberculosis* (leporine isolate)	$5.55x10^7$cfu	3[c]

[a]The five dams were necropsied six weeks after the inoculation of their kits
[b]Three rabbits were killed prior to the six month time point (see section 5.6 below)
[c]One rabbit was killed prior to the six month time point (see section 5.6 below)

The kits were kept as a litter with their dams until six week pi, when they were weaned and thereafter caged separately. At weaning, the dams of the five litters were killed by an

intravenous injection of barbiturate solution, and samples taken for histopathological analysis and culture (Table 5-7).

The experimental plan incorporated two post mortem dates, six and 12 months pi, with 12 rabbits to be killed and examined at each time point. Samples of duodenum, jejunum, mid ileum, terminal ileum, sacculus rotundus, appendix, colon, and mesenteric lymph node were taken during the post mortem (Plate **5,10**), and a representative sample of each tissue placed in 10% formal saline and processed as described for histopathological analysis (see sections 2.1.5 and 2.1.6), with the remainder pooled and cultured (described in section 2.1.2). A sample of each liver, spleen and right kidney was also taken for histopathological analysis (Table 5-7).

Table 5-7 Samples collected from each rabbit at post mortem examination

	culture	histopathology
duodenum	✔	✔
jejunum	✔	✔
mid ileum	✔	✔
terminal ileum	✔	✔
sacculus rotundus	✔	✔
MLN	✔	✔
appendix	✔	✔
colon	✔	✔
right kidney		✔
liver		✔
spleen		✔

5.3.2 Results

Four rabbits died during the incubation period of the experiment (details in section 5.6), consequently, only eight were killed and examined after six months - four from group Co_r, one from group Bo_r, and three from group Ra_r. At the time of writing, five rabbits remain in the control group, four in the group Bo_r and four in group Ra_r, and will be killed and necropsied 12 months pi. No rabbit developed diarrhoea during the first six months of the experiment, however, one developed gradual weight loss, necessitating euthanasia at 10 weeks (see section 5.6). No evidence of *M.a. paratuberculosis* infection was identified at post mortem examination or by histopathological or microbiological analysis of tissues from this animal.

5.3.2.1 Post mortem examination

No abnormalities were noted on gross examination of any rabbits. All carcasses had abundant intra-abdominal fat.

5.3.2.2 Histopathology

No AFB were identified in any section, and no pathology suggestive of *M.a. paratuberculosis* infection was noted.

5.3.2.3 Microbiology

No bacterial colonies were isolated from any tissue after the standard four month incubation period. The slopes will be incubated for a total of 15 months.

5.3.3 Discussion.

Rabbits experimentally inoculated with a bovine isolate of *M.a. paratuberculosis* did not develop signs of paratuberculosis, nor was any infection evident using either culture or histopathological examination of intestinal tissues. Therefore, this experiment provides no proof that an isolate of *M.a. paratuberculosis* derived from cattle is capable of infecting rabbits. However, no mycobacteria were isolated from the "positive control" group of rabbits that were inoculated with a rabbit-derived isolate of *M.a. paratuberculosis*, and no histopathological changes were identified. Thus, infection does not appear to have been established and it is not possible to determine, from the results of this experiment, whether the bovine isolate of *M.a. paratuberculosis* is pathogenic to rabbits, or if it exhibits comparable virulence to the leporine isolate when inoculated into rabbits.

It is not clear why rabbits from groups Bo_r and Ra_r failed to develop *M.a. paratuberculosis* infection. The dose used in the inoculum was considered sufficient, and the experimental protocol mimicked the trickle infection thought to be representative of the situation experienced by free-living rabbits. Two previous reports of experimental *M.a. paratuberculosis* infection of young rabbits describe a similar experimental design, with comparable inoculation doses, and report infection rates of 62% and 75% (Mokresh *et al* 1989, Mokresh and Butler 1990). However, recovery of *M.a. paratuberculosis* organisms from the tissues of the experimentally infected rabbits in these studies was possible only after incubating the slopes for up to 15 months, and the rabbits in both experiments were infected with intestinal coccidiosis. Whether

intestinal coccidiosis infection, common amongst the free-living rabbits examined in chapter 3, predisposes rabbits to *M.a. paratuberculosis* infection is not known. In response to these previous reports, the bacterial slopes from the study described in this chapter will be incubated for a total of 15 months.

The results of this experiment have a number of possible explanations. The rabbits in this experiment may have been infected, but the infection not detected. The pathogenesis of rabbit paratuberculosis is still unclear, therefore it is possible that the organism undergoes a lengthy incubation phase in the rabbits in a tissue or region not examined in this study. Alternatively, the rabbits may have been free from infection. Free-living rabbits may be more susceptible to *M.a. paratuberculosis* infection than the New Zealand white laboratory-bred animals, possibly as a consequence of the more challenging lifestyle of the wild rabbits. The lack of infection noted in the experimentally inoculated rabbits may be because, in contrast to the situation in ruminants, neonatal rabbits may be particularly resistant to infection with *M.a. paratuberculosis*, with free-living animals becoming infected only when older. It is also possible that the most common means of transmission of *M.a. paratuberculosis* between rabbits may not be via the oral route, but vertically, with horizontal transmission rare or non-existent.

5.4 Conclusion

Paratuberculosis was induced in calves inoculated with either a bovine or leporine isolate of *M.a. paratuberculosis*, indicating the potential for transmission of disease from rabbits to cattle. However, it was not possible to replicate the florid lesions seen in naturally infected, free-living rabbits (described in chapter 3) by experimental means. Both the dams and kits in the study reported here exhibited remarkable resistance to *M.a. paratuberculosis* infection, suggesting that oral inoculation of rabbits with *M.a. paratuberculosis* is insufficient for infection to occur, and that further, as yet unidentified factors, are involved in the aetiology of rabbit paratuberculosis.

5.5 Details of calves treated while on study

Animal no.	Group	Week of trt	Suspected problem	Treatment given
1543	Bo_b	2	septic arthritis	Given 4ml synulox[1] (batch no 80769333 exp date 03-2000) SC daily for 5d, and 3ml finadyne[2] (batch no. CNXC 7033, exp date Aug00) IV for 3d.
1547	Bo_b	2	omphalitis	4ml synulox[1] SC (batch no 80769333 exp date 03-2000) and 3ml finadyne[2] (batch no. CNXC 7033, exp date Aug00) IV for 3d
1522	Ra_b	8	pneumonia	Given 4ml 10% baytril[3] (batch no BN ss3043 exp date 02 2000) SC and 5ml finadyne[2] (batch no. CNXC 7033, exp date Aug00) IV for 3d
1526	Ra_b	8	bloat	Given 4ml finadyne[2] IV (batch no. CNXC 7033, exp date Aug00) on d1 and d3

[1]Synulox® Ready To Use injection (Pfizer Ltd) containing 35mg/ml clavulanic acid as pottasium clavulanate PhEur and 140mg/ml amoxycillin as amoxycillin trihydrate PhEur.
[2]Finadyne® solution (Schering-Plough Animal Health) containing 50mg flunixin, as flunixin meglumine BP (Vet) and 5mg phenol Ph Eur as a preservative, per ml.
[3]Baytril® 10% injection (Bayer Plc) containing 100mg/ml enrofloxacin and 30mg/ml n-butyl alcohol as a preservative

5.6 Details of rabbits treated while on study

Group no.	Number of animals affected	Week of treatment	Suspected problem	Treatment given
Bo_r	2	2	mismothering	euthanasia[5]
Bo_r	1*	4	corneal ulcer	topical vistamethasone[1] qid for 48 hours, topical atropine[2] tid for 5 days, tiacil[3] tid for 14 days
Bo_r	1	10	gradual weight loss, cause unknown	euthanasia[5]
Bo_r	1*	20	eye infection	aureomycin[4] bid for 4 days
Ra_r	1	20	anorexia, cause unknown	euthanasia[5]

* Same animal - eye infection reoccured
[1]vectamethasone® (Martindale Pharmaceuticals Ltd), containing betamethasone sodium phosphate 0.1% [w/v], neomycin sulphate BP 0.5% [w/v], thiomersal BP 0.01% [w/v].
[2]atropine sulphate, non-proprietary preparation
[3]tiacil® ophthalmic solution (Virbac ltd) containing 3mg gentamicin sulphate, 6mg trometamol, and 5mg disodium edetate (EDTA) in 1 ml.
[4]aureomycin ophthalmic ointment (Fort Dodge Animal Health) containg 1% chlortetracycline hydrochloride
[5]all rabbit were euthanased using intravenous or intraperitoneal barbiturate injection.

6 Early Pathological and Immunological Changes in *M.a. paratuberculosis* Infection.

6.1 Introduction

The immunological changes that occur during the initial stages of *M.a. paratuberculosis* infection may be crucial in determining whether the outcome of the challenge is expulsion of the organism and full recovery, or persistence of the bacterium and chronic subclinical infection. A clearer understanding of how animals resist initial infection could lead to the development of more efficient vaccines.

The gut associated lymphoid tissues (GALT), consisting of JPP, IPP and MLN, are thought to play a central role in the early pathogenesis of paratuberculosis. Evidence suggests that the organism crosses the mucosa of the gut via M cell endocytosis, and enters the underlying lymphoid follicle, where the earliest evidence of *M.a. paratuberculosis* infection can be found (Momotani *et al* 1988)(discussed in section 1.7.1 above). It is in these areas during the first four to eight weeks following infection that the critical events of *M.a. paratuberculosis* infection are likely to occur.

While early pathological lesions have been described by a number of workers (see section 1.7.1.2 above), there have been few *in vivo* experiments investigating the immunological changes immediately following *M.a. paratuberculosis* infection. One such study analysed the immune responses of young lambs over eight weeks following oral inoculation with *M.a. paratuberculosis* (Begara-McGorum *et al* 1998). This work reported higher numbers of $CD8^+$ and $CD2^+$ lymphocytes, as well as increased levels of the cytokines TNF-α and GM-CSF in infected lambs, suggesting that, similar to other mycobacterial diseases, the early response to *M.a. paratuberculosis* infection is predominately cell mediated (Dannenberg, Jr. 1991, Orme 1993).

The majority of the immunological investigations into paratuberculosis have been restricted to clinical disease. As a result, there remains much to be elucidated about the early immunological events of *M.a. paratuberculosis* infection, including the potential role of $\gamma\delta$ T cells and CD1

molecules. Gamma delta T cells are thought to play an important role at mucosal surfaces as part of the first line of defence against infectious agents including intracellular pathogens such as *Salmonella*, *Listeria* and *Mycobacteria* (see section 1.7.4 above). Gamma delta T cells increase in number in response to a range of mycobacterial pathogens (Table 1-2), however, their role in defence against *M.a. paratuberculosis* infection is unknown. CD1 is a cell surface glycoprotein which acts as an APM, presenting lipid antigens, including mycobacterial cell wall components, to T lymphocytes (Beckman *et al* 1994, Sugita *et al* 1998, Burdin and Kronenberg 1999) (section 1.7.4).

6.2 Experiment A

6.2.1 Hypothesis

The aim of this experiment was to reproduce *M.a. paratuberculosis* infection experimentally in young lambs, to allow the characterisation of the pathological, bacteriological and immunological responses. As part of this investigation, the hypothesis that CD1 molecules recognise *M.a. paratuberculosis* antigens and present them to the abundant γδ T cells in the neonatal ruminant GALT, forming part of the primary immune response against the pathogen, was tested.

In addition to attempting to clarify the early immunopathogenesis of paratuberculosis, the comparative virulence of different strains of *M.a. paratuberculosis* was investigated. The current uncertainty regarding the species-specificity of different strains of *M.a. paratuberculosis* and consequently the extent of interspecies transmission of the organism hamper a thorough understanding of the epidemiology of this disease. Thus, an ovine, bovine and cervine isolate of *M.a. paratuberculosis* were inoculated into lambs, and the resultant changes compared.

6.2.2 Materials and Methods

Dams and their offspring were tested for the presence of *M.a. paratuberculosis* antibodies prior to the experiment, using an ELISA as described earlier (Begara-McGorum *et al* 1998). The inocula were prepared (section 2.1.1 above), and included strains derived from a case of cervine paratuberculosis (JD88/107), ovine paratuberculosis (51/91) and bovine paratuberculosis (F13).

6.2.2.1 Animal work

Forty lambs (Suffolk x Texel) of either sex, aged between eight and 19 days old, were conventionally housed in indoor pens with their dams for the duration of the experiment. Ten lambs were randomly allocated to four equal groups, group Co was designated the control group, and received 5ml of sterile PBS at each inoculation. Lambs from group Ce were infected with the cervine isolate, group Ov with the ovine isolate, and group Bo with the bovine isolate. Lambs in groups Ce, Ov and Bo were each dosed orally with approximately $1x10^9$ cfu of *M.a. paratuberculosis* suspended in 5ml of sterile PBS on days 0, 2 and 4. Retrospective counts (section 2.1.4 above) on the inoculum were performed to determine more accurately the

infective dose (Table 6-1). The lambs were killed with intravenous barbiturate solution 28 days after the first inoculation.

Table 6-1 The number of organisms in the inoculum given to each animal, as determined by retrospective viable counts.

	cfu/ml	cfu/dose	total number of cfu
Group Co	0	0	0
Group Ce	$5.5x10^6$	$2.8x10^7$	$8.4x10^7$
Group Ov	$1.3x10^7$	$6.5x10^7$	$1.95x10^8$
Group Bo	$3.85x10^7$	$1.9x10^8$	$5.7x10^8$

6.2.2.2 Microbiology

Samples of MLN and IPP were collected aseptically from all lambs at the time of post mortem examination, and cultured on modified Middlebrooks 7H11 medium (section 2.1.2).

6.2.2.3 Histopathology

At post mortem examination, tissues were inspected grossly, and samples of terminal ileum, JPP, IPP, ICV, MLN, ICLN, retropharangeal and submandibular lymph nodes were collected for routine histopathological analysis (sections 2.1.5 and 2.1.6).

6.2.2.4 Fluorescent antibody cell sorting (FACS) method

Lymphocytes were collected and prepared for FACS analysis as described earlier (section 2.1.10). Single colour flow cytometric analysis was performed on the lymphocytes, using monoclonal antibodies against the ovine CD4, CD8 and Ig light chain molecule (Table 6-2). Results were expressed as a percentage of positively stained cells in a sample population of 10 000 individual cells.

Table 6-2 Details of monoclonal antibodies used for FACS analysis on the JPP, IPP and MLN.

Monoclonal Antibody	Antigen identified	Reference
IAH-CC15	$WC1^+$(γδ T cells)	(Wijngaard *et al* 1992)
IAH-CC20	Ovine CD1	(Howard *et al* 1993)
17D	Ovine CD4	(Mackay *et al* 1988)
7C2	Ovine CD8	(Mackay *et al* 1988)
VPM8	Ovine light chain Ig (B cells)	(Bird *et al* 1995)

6.2.2.5 Immunohistochemistry

Estimates were made of the number of γδ T cells and CD1 molecules present by immunohistochemical labelling. Samples of JPP, IPP and MLN were collected at necropsy, snap frozen in a slurry of dry ice and isopentane (Sigma-Aldrich) for approximately 30 seconds and stored at -70^{o}C until required. Cryostat sections 6μm-thick were cut from the frozen tissues, and labelled as described earlier in section 2.1.9.

γδ T cells were labelled with monoclonal antibody (mAb) IAH-CC15 which recognises the surface antigen WC1 (Wijngaard *et al* 1992, Crocker *et al* 1993) expressed on γδ T cells which lack CD2, CD4 and CD8 molecules. CD1 molecules were labelled with mAb IAH-CC20 which has a CD1b-like pattern of reactivity, belonging to the cluster BoCD1w2 (Howard *et al* 1993).

Once the sections had been labelled with either CC15 or CC20, the slides were examined microscopically, and the number of positively labelled cells counted. Sections from JPP and IPP were divided into four areas - villous, dome, interfollicular, and follicular (Plates **6,1-6,2**). The number of positive cells per approximately 100 total cells was calculated in at least two separate locations for each area on each slide. Thus for each animal, a minimum of eight counts was obtained for each tissue and each antibody. Sections from MLN were examined under 400x magnification, and the number of positive cells per high power field counted. At least five high powered fields were counted in both the cortex and the medulla.

6.2.2.6 Statistics

The raw data from the FACS results was transformed to $\log_{10}$ and an ANOVA performed to determine statistical differences between groups and calculate p-values. However, it was necessary to use Generalised Linear Mixed Models (GLMM), rather than simpler statistical analyses, for the γδ T cell and CD1 molecule data because of the non-independence of counts drawn from the same animal (Brown and Prescott 1999). Jejunal Peyer's patch and IPP counts were analysed using a GLMM with a Binomial error distribution, whereas the MLN data was analysed by fitting a GLMM with a Poisson error distribution. This difference reflected the different counting strategy used for the MLN data. For data from JPP and IPP, a separate analysis was carried out for each combination of tissue, antibody and area. The data were the number of positive cells from the total number of cells observed during each count. Accordingly, in each analysis, a GLMM with binomial variation and a logit link function was fitted, modelling between-sheep and residual variation as random effects and infective status as a fixed effect. Tests of significance were carried out on the logit scale.

For data from the MLN tissue the data were numbers of positive cells. A GLMM with Poisson variation and a logarithmic link function was fitted, again modelling between-sheep and residual variation as random effects and infective status as a fixed effect. Tests of significance were carried out on the logarithmic scale.

6.2.3 Results

6.2.3.1 Post mortem examination, histological and microbiological results

One lamb showed gross evidence of intestinal disease at post mortem examination. It had been inoculated with the cervine isolate of *M.a. paratuberculosis* and was dull, dehydrated and appreciably smaller than its cohorts. The wall of the ileum of this animal was thickened and the mucosa markedly corrugated. The mesentery leading from the ileum contained swollen lymphatic and blood vessels, however, the associated mesenteric lymph nodes were grossly normal. On histopathological examination, there was marked erosion of the epithelium of the ileum, with polymorphonuclear exudate present in the intestinal lumen (Plates **6,3-6,4**). The underlying lymphoid tissue appeared normal. No AFB, macrophage granulomata, or epithelioid cells were noted, and no *M.a. paratuberculosis* organisms were cultured from the tissues,

therefore it was deemed unlikely that these lesions were a result of *M.a. paratuberculosis* infection. All data from this animal were excluded from the experimental analysis.

In the remaining 39 animals, no gross lesions were evident at post mortem, no changes consistent with *M.a. paratuberculosis* infection were noted during histopathological examination, no AFB were identified in tissue sections, and no bacteria were grown from any tissue sample.

6.2.3.2 Fluorescent antibody cell sorting (FACS) analysis

Results are listed in Table 6-3 and depicted in Figure 6-1. Two statistically significant changes were apparent in the PBL of the inoculated lambs - an increase in the $CD4^+$ lymphocytes and a decrease in the B cells. The rise in $CD4^+$ lymphocytes was apparent also in the JPP, while the B cell decrease was present in the IPP. No statistically significant changes were noted in the lymphocyte subsets of the MLN.

Table 6-3: Results of FACS analysis from the PBL, JPP, IPP, and MLN of the four groups of lambs.

Data presented shows the mean of the % stained cells with the standard error of the mean in parenthesis. Significant differences between individual infected groups and the control group were determined using analysis of variance. Probability values are given at the foot of each section.

PBL	$CD4^+$	$CD8^+$	B cells
Group Co	14.1 (1.65)	21.5 (5.81)	50.6 (13.84)
Group Ce	25.1 (4.84)[1]	22.5 (5.75)	42.2 (11.08)
Group Ov	21.1 (2.24)[2]	25.2 (5.06)	36.1 (9.96)[4]
Group Bo	26.3 (4.13)[3]	24.2 (4.54)	32.0 (9.15)[5]

1. p=0.016, **2**. p= 0.010, **3**. p=0.002, **4**. p=0.031, **5**. p=0.006

JPP	$CD4^+$	$CD8^+$	B cells
Group Co	19.4 (1.48)	20.1 (3.10)	40.8 (3.86)
Group Ce	25.4 (1.78)[6]	21.1 (3.40)	36.9 (3.91)
Group Ov	28.7 (5.10)[7]	24.3 (2.83)	42.2 (4.45)
Group Bo	24.6 (4.03)	17.8 (3.78)	33.0 (2.96)

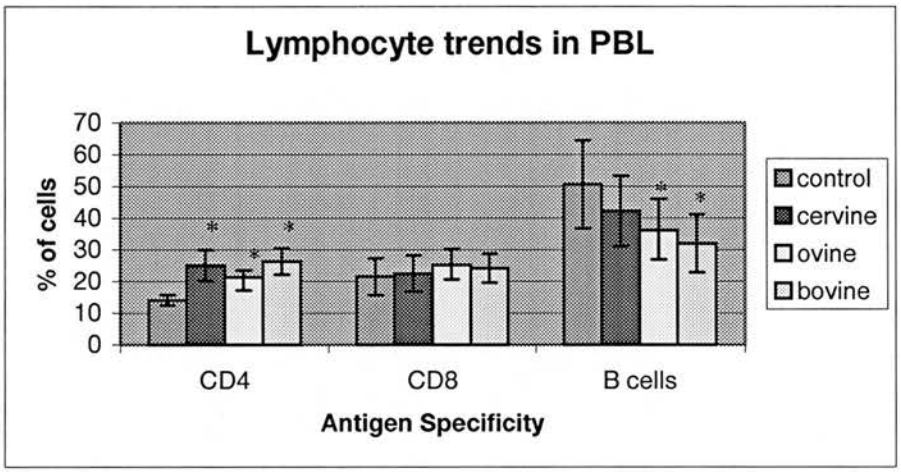
6. p= 0.019, **7**. p= 0.068.

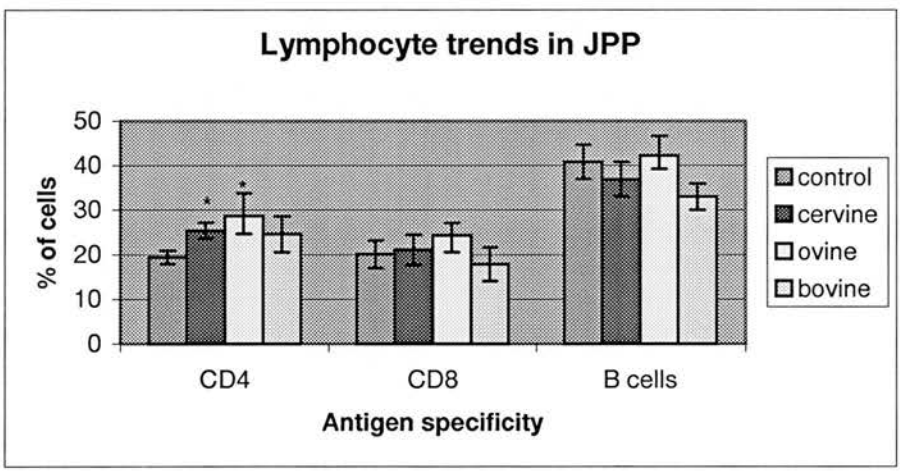
IPP	$CD4^+$	$CD8^+$	B cells
Group Co	11.2 (1.5)	8.7 (1.6)	90.9 (0.9)
Group Ce	13.5 (2.6)	8.0 (1.6)	82.3 (2.3)[8]
Group Ov	14.2 (2.4)	13.9 (5.0)	89.4 (1.0)
Group Bo	9.1 (1.2)	11.0 (2.2)	80.4 (3.7)[9]

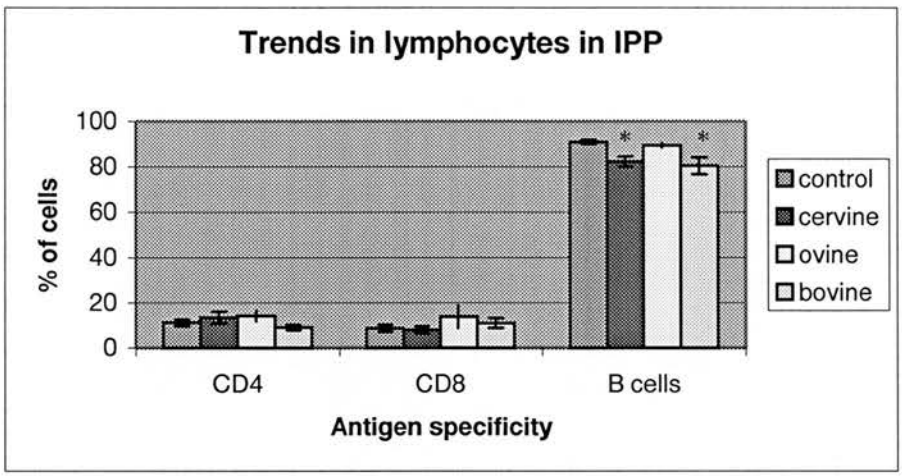
8. p=0.003, **9**. p=0.012.

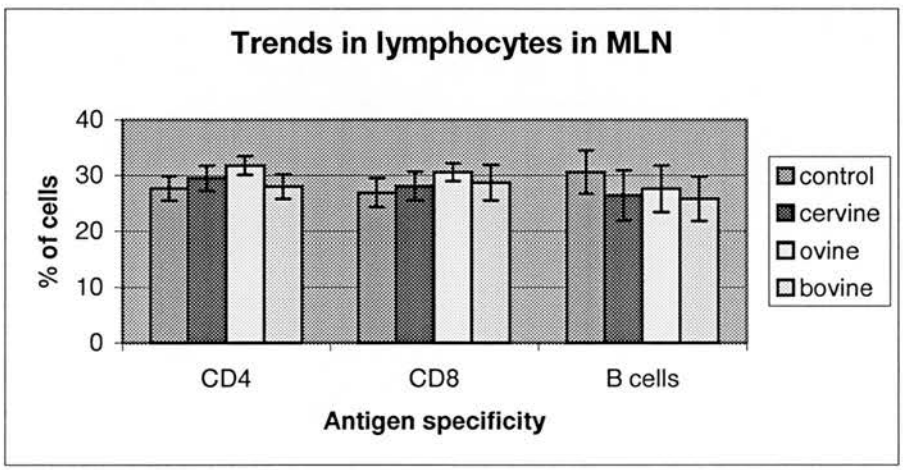
MLN	$CD4^+$	$CD8^+$	B cells
Group Co	27.7 (2.2)	26.9 (2.6)	30.6 (3.9)
Group Ce	29.5 (2.3)	28.1 (2.6)	26.4 (4.5)
Group Ov	31.8 (1.7)	30.6 (1.6)	27.6 (4.2)
Group Bo	28.0 (2.2)	28.7 (3.2)	25.8 (4.0)

Figure 6-1 Graphical representation of Table 6-3, comparing the lymphocyte subsets in the PBL, JPP, IPP, and MLN in the four groups of lambs, with significantly different values marked by *






Trends in lymphocytes in IPP
100
80
60
40
20
0
% of cells
*
*
CD4
CD8
B cells
Antigen specificity
control
cervine
ovine
bovine


Trends in lymphocytes in MLN
40
30
20
10
0
% of cells
CD4
CD8
B cells
Antigen specificity
control
cervine
ovine
bovine

6.2.3.3 Immunohistochemistry

Five lambs from group Co and five lambs from group Ce (cervine group) were analysed by immunohistochemistry. On examination of the frozen tissues, samples of the JPP were found to be missing from one control and one infected lamb. Consequently, the data for the JPP is calculated from eight, not 10 animals. Minimal numbers of γδ T cells and CD1 molecules were seen in the follicular areas in both the JPP and IPP, consequently, this data was not included in the analysis. Similarly, CD1 molecules were very rarely noted in the villi of control and infected tissue, and were also excluded from analysis. Examples of labelled cells can be seen in Plates **6,5** and **6,6**.

The results of the statistical analysis are summarised in Table 6-4 and Table 6-5. Meaningful comparisons of averages from the raw data cannot be made because of potential bias from the unequal numbers of counts being made from each animal. Instead, the GLMM fits a statistical model for the probability of each observed cell being positive. The results of these models were "back-transformed" to provide estimates for the average percentage of positive cells observed in IPP and JPP tissues, and the average number of positive counts in MLN in both infected and uninfected animals. The GLMM provided an estimate of the standard error of the difference between these averages on the transformed scale (either logit or logarithmic, as appropriate), from which a p-value can be calculated. The Satterthwaite approximation was used to derive the appropriate degrees of freedom for use in these tests.

Table 6-4 Summary of the GLMM statistical analysis of immunohistochemistry results from the JPP and IPP, showing the rise in the number of γδ T cells in both tissues.

The data is presented as the estimated mean percentage of cells counted as positive in control and inoculated groups, the standard error of the difference between these percentages, and the corresponding p-values.

Ab	Tissue	Area	Control Group[a]	Inoculated Group[a]	s.e. of difference[b]	p-value
γδ	JPP	villous	5.4	7.0	0.256	0.33
		dome	3.2	6.9	0.256	0.03*
		interfoll.	2.7	4.7	0.259	0.08†
	IPP	villous	5.3	9.2	0.218	0.03*
		dome	2.9	5.7	0.320	0.06†
		interfoll.	2.2	4.0	0.322	0.10†
CD1	JPP	villous	0.1	0.2	1.814	0.62
		dome	5.4	8.9	0.446	0.28
		interfoll.	5.4	3.5	0.278	0.17
	IPP	villous	NA	NA	NA	NA[c]
		dome	2.6	2.8	0.337	0.89
		interfoll.	3.8	5.8	0.327	0.22

[a]Estimated mean percentage of positively stained cells

[b]On logit scale

[c]Model failed to converge, due to lack of differences between groups

*p<0.05

†p<0.10

Table 6-5 Summary of the GLMM statistical analysis of immunohistochemistry results from the MLN.

The data is presented as the estimated mean count per high powered field of positive cells in control and inoculated groups, the standard error of the difference between these counts, and the corresponding p-values.

Ab	Tissue	Area	Control group[a]	Inoculated group[a]	s.e. of difference[b]	p-value
γδ	MLN	cortex	27.3	45.9	0.393	0.23
		medulla	22.8	36.5	0.295	0.15
CD1	MLN	cortex	9.1	15.7	0.493	0.30
		medulla	3.4	3.4	0.342	0.94

[a]Estimated mean count of positively stained cells per high powered field
[b]On logarithmic scale

An increase in the numbers of γδ T cells was noted in the inoculated group in all three areas (villous, dome and interfollicular) of the JPP and IPP. Using the GLMM model, it was found that, with the exception of the villous area of the JPP, these increases were either statistically significant at the 5% level, or close to such significance ($p<0.1$). The γδ T cell numbers were higher in both the cortex and medulla of the MLNs from the inoculated group, but these differences were not statistically significant (Table 6-5).

There were no significant differences in the numbers of CD1 molecules between the two groups in any area of the JPP, IPP or MLN. There was a small decrease in the number of CD1 cells in the interfollicular area of the JPP in inoculated animals, but this difference was not statistically significant ($p=0.17$).

6.2.4 Discussion

This experiment investigated immunological and pathological changes in young lambs exposed to *M.a. paratuberculosis.* Particular attention was paid to the areas thought to be most important in early interactions with *M.a. paratuberculosis* organisms - the JPP, IPP and MLN.

There were no pathological changes detected in the lambs, and no *M.a. paratuberculosis* organisms cultured from tissues. It is not clear why this occurred, since the previous study, utilising a very similar infection model, reported pathological changes indicative of *M.a. paratuberculosis* infection in four of eight inoculated lambs, and isolation of *M.a. paratuberculosis* from the tissues of three of eight inoculated lambs (Begara-McGorum *et al* 1998). Early changes in *M.a. paratuberculosis* infection are often very subtle (Nisbet *et al* 1962, Perez *et al* 1996, Sigurdardottir *et al* 1999) and may have been overlooked in the present experiment, however, the negative histology and bacteriology results also could be interpreted as a lack of proof that a true *M.a. paratuberculosis* infection was established. The immunological changes noted may therefore be the result of successful defence against *M.a. paratuberculosis*, with all organisms repelled, rather than an initial immunological response to chronic mycobacterial infection.

There was no indication in this experiment that the different isolates of *M.a. paratuberculosis* differed in virulence for the lambs. However, since no conclusive evidence of infection was identified, this finding must be regarded as preliminary.

The FACS analysis provided evidence of a cmi response, with a higher proportion of $CD4^+$ lymphocytes and lower proportion of B cells present in inoculated lambs. This would indicate that, along with other mycobacterial diseases, cmi provides the earliest responses to *M.a. paratuberculosis* infection (Dannenberg, Jr. 1991, Orme 1993, Cooper and Flynn 1995, Schaible *et al* 1999). The lymphocyte data suggests that the immune response differs between the GALT areas, with the increase in $CD4^+$ lymphocytes present only in the JPP, the decrease in the B cells present only in the IPP, and no changes evident in any lymphocyte subset in the MLN. The possibility that the different GALT areas act independently has been suggested previously (Begara-McGorum *et al* 1998).

One of the most interesting findings of this experiment was the increase in the number of γδ T cells in the JPP, IPP and MLN in animals exposed to *M.a. paratuberculosis,* suggesting that these cells may be an important component of the early host immune response. There have been few previous studies investigating the response of ruminant γδ T cells to mycobacterial infection. Levels of γδ T cells in the peripheral blood of calves initially decreased after experimental infection with *M. bovis*, and then increased, suggesting localisation to developing lesions, followed by clonal expansion (Pollock *et al* 1996). Our results would support this general premise. Other workers failed to find evidence of increased activity of γδ T cells in red deer eight weeks after experimental infection with *M. bovis*, but suggested that this was due to the length of time elapsed between infection and sampling (Cross *et al* 1996). No statistically significant change was seen in the numbers of γδ T cells in the GALT of young lambs experimentally infected with *M.a. paratuberculosis* in a previous experiment (Begara-McGorum *et al* 1998), however, this study pooled the results from lambs examined from three days to eight weeks pi, which may have masked an early γδ T cell response.

One factor crucial in the elucidation of the role of the increased numbers of γδ T cells is the identity of the APM involved in the putative γδ T cell-*M.a. paratuberculosis* interaction. We found no evidence that CD1-expressing APCs were up or down regulated by *M.a. paratuberculosis* exposure, and thus no suggestion that CD1 acts as an APM for γδ T cells. One interesting result, however, was a decrease in the number of $CD1^+$ cells in the interfollicular area of the JPP in the infected group of lambs. Infection of APCs with *M. tuberculosis* has been shown to downregulate the expression of human CD1b, consistent with an immune evasion

mechanism (Stenger *et al* 1998). Although the decrease observed in the number of CD1 cells in the JPP of the infected group in our study did not reach statistical significance, it may represent a mild *in vivo* illustration of the reported downregulation mechanism employed by *M. tuberculosis*.

Future work into possible γδ T cell interactions should attempt to clarify the APC and APM involved. While the work described in this chapter found no increase in the number of $CD1^+$ cells in response to *M.a. paratuberculosis* inoculation, our investigation used antibody only to the CD1b molecule, therefore other CD1 proteins, particularly the group 2 proteins, such as CD1d, should be investigated. It is possible that CD1 molecules do present *M.a. paratuberculosis* antigens to γδ T cells, but the immunohistochemical labelling technique used in this experiment was not sufficiently sensitive to detect the mild changes in CD1 molecule numbers. Other techniques such as FACS analysis, realtime PCR, or the use of microarrays may reveal these alterations. Dual labelling techniques could enable a correlation between the distribution of γδ T cells and CD1 molecules to be detected, strengthening the hypothesis that they act together in the immune response to *M.a. paratuberculosis* infection.

It is of course possible that an alternative APM is involved in the γδ T cell expansion. The Nramp protein and HSP60 have both been suggested by other workers to act as APMs to γδ T cells (Kaufmann 1996, Boom 1999). Some investigations have even indicated that γδ T cells can recognise antigens without the assistance of an APM (Morita *et al* 1995).

The role of the increased number of γδ T cells in the infected lambs' GALT therefore remains unclear - whether, in these specialised lymphoid compartments, they assist the host to combat the infection, or whether they suppress other host immune responses, and thus aid *M.a. paratuberculosis* to evade the immune system and achieve a persistent state in the lymphoid tissue, is a matter of conjecture. The third possibility is, of course, that the increase in γδ T cells is merely a reaction to foreign material in the host's gut, is not specifically directed against the *M.a. paratuberculosis* organism, and has no effect on *M.a. paratuberculosis* infection.

6.3 Experiment B

6.3.1 Hypothesis

Previous work (detailed in section 6.2 above) revealed for the first time that γδ T cells in the GALT of the gut increase in response to *M.a. paratuberculosis* inoculation, suggesting that this T cell subset may play a role in the host's immune response to the pathogen. In order to link the rise in γδ T cells with bacteriological and pathological evidence of *M.a. paratuberculosis* infection, and to investigate the stimuli responsible for the increase, the responses of young lambs to oral inoculation with live and heat-killed *M.a. paratuberculosis* were compared. Thus if an increase in γδ T cells was detected only in the animals inoculated with live *M.a. paratuberculosis* organisms, this would suggest that the rise is due to a specific host-*M.a. paratuberculosis* interaction, and is not simply the result of foriegn material being detected in the gut.

6.3.2 Materials and Methods

The dams and their lambs were tested for the presence of *M.a. paratuberculosis* antibodies prior to the experiment, using an ELISA as described earlier (Begara-McGorum *et al* 1998). The inocula were prepared as detailed previously in section 2.1.1. Mycobacteria were inactivated by heat treatment, when a *M.a. paratuberculosis* cell suspension in PBS was adjusted to McFarland standard 2.8 and aliquotted into 1.5ml screw-topped microfuge tubes. The tubes were completely immersed in a waterbath at 80^0C for 30 minutes, then removed and allowed to cool to room temperature. Inactivated cells were pooled before use.

6.3.2.1 Animal work

Thirty lambs aged between 17 and 30 days old, mostly Dorset with a small number of Dorset x Suffolk animals, were randomly allocated to three groups, each of 10 lambs. The lambs were conventionally housed with their dams for the duration of the experiment, with each group housed seperately. Lambs in group Hk were orally dosed on days 0, 2 and 4 with approximately $1x10^9$ cfu of heat killed *M.a. paratuberculosis* (cervine isolate JD88/107) suspended in 5ml of PBS. The 10 lambs in group Li received an oral dose of approximately $1x10^9$ cfu of live *M.a. paratuberculosis* (cervine isolate JD88/107) suspended in 5ml of PBS on days 0, 2 and 4, while group Co was designated the control group and received 5ml of PBS at each inoculation.

Animals were killed by intravenous barbiturate solution and necropsied 28 days after the first inoculation. Retrospective count data revealed that each animal in group Li received three 5ml doses, each containing $2.1x10^8$ cfu of *M.a. paratuberculosis*. A sample of each heat killed inoculum was also cultured, and no viable organisms obtained.

6.3.2.2 Microbiology

Samples of MLN and IPP were collected aseptically at the time of post mortem examination from all 30 lambs, and cultured on modified Middlebrooks 7H11 medium (section 2.1.2). All isolates grown were confirmed by the PCR assay as containing IS900 (section 2.1.15).

6.3.2.3 Histopathology

Samples of terminal ileum, JPP, IPP, ICV, MLN, ICLN, and popliteal lymph node were collected for routine histopathological analysis.

6.3.2.4 Immunohistochemistry

Samples of JPP, IPP, and MLN were collected at necropsy as described earlier in section 6.2.2.5 and stored at $-70^{o}C$.

6.3.2.5 Fluorescent antibody cell sorting (FACS) method

A sample of venous blood was collected just prior to the first inoculation, and then at weekly intervals, including a sample just prior to post mortem. FACS analysis was carried out on a sample of MLN, JPP, IPP and popliteal lymph node as described earlier in section 6.2.2, with the addition of CD1 and γδ T cell mAbs (Table 6-2).

6.3.3 Results

6.3.3.1 Post mortem

No gross abnormalities were detected in any animal during post mortem examination.

6.3.3.2 Histopathology

Multiple small, non-encapsulated but well defined granulomata were identified deep in the lymphoid follicles of the JPP in four lambs from group Li ("live-infected"). These granulomata consisted of swollen macrophages, the occasional giant cell, admixed fibroblasts, and contained a centrally located small focus of dystrophic calcification. A small number of infiltrating

lymphocytes were noted in the granulomata, but no polymorphonuclear cells or AFB were identified in either the granulomata or surrounding lymphoid tissue (Plates **6,7-6,9** and **6,11-6,12**). No paratuberculosis-like lesions were noted in the lamina propria or epithelium of the JPP, or in any other tissues examined. The results of the histopathological examination are summarised in Table 6-6.

Individual degenerating, amorphous cells were found scattered in the lymphoid follicles of the JPP in four lambs from group Co, five lambs from group Hk, and two lambs from group Li. These cells were not associated with granulomata, giant cells, swollen macrophages or any other similar structure and were not regarded as sequelae of *M.a. paratuberculosis* infection (Plate **6,10**).

No histopathological lesions suggestive of early *M.a. paratuberculosis* infection were noted in any animal from either group Co or Hk.

Evidence of intestinal coccidiosis was seen in animals from all three groups. The extent of the infection ranged from mild to moderate, with a varying number of coccidial oocysts identified in the intestinal epithelium of the JPP, IPP and terminal ileum (Plate **6,13**). The infection was never severe enough to produce clinical signs, gross lesions, or marked histopathological changes, and the extent and severity of the lesions was comparable between the three groups of lambs. The coccidiosis infection in each lamb was graded subjectively from 0 (no evidence of infection) to 3 (moderate level of infection).

6.3.3.3 Microbiology

Cultures of *M.a. paratuberculosis* (Table 6-6) were obtained from IPP tissues from two lambs, both of which were in group Li, the live infected group. No other tissue yielded *M.a. paratuberculosis* isolates.

6.3.3.4 Fluorescent antibody cell sorting (FACS) analysis

No changes between groups were identified in the subsets of the PBL taken at weekly intervals during the incubation period. FACS analysis of the tissues collected at post mortem examination revealed no trends in any lymphocyte subset. The data were analysed using both coccidiosis score and presence of paratuberculosis-like lesions as a confounding factor, but no significant

changes were identified. A summary of the FACS data from this experiment is listed in appendix one .

Table 6-6 Number of animals in each group with microbiological or histopathological changes four weeks after oral inoculation with *M.a. paratuberculosis*.

	Total number of animals in group	Lesions present[1]	Culture positive[2]	Coccidiosis present[3]
Group Co	10	0	0	8
Group Hk	10	0	0	6
Group Li	10	4	2	8

[1]Lesions consistent with early *M.a. paratuberculosis* infection
[2]*M.a. paratuberculosis* organisms were isolated from tissues collected at post mortem examination
[3]Coccidial oocysts were identified in sections of intestine

6.3.4 Discussion

The results from this experiment were encouraging, as *M.a. paratuberculosis* organisms were recovered from, and lesions consistent with paratuberculosis noted in, tissues from "live-inoculated" animals, indicating that *M.a. paratuberculosis* infection was present. No *M.a. paratuberculosis* isolates were recovered from, or similar lesions detected in, animals from the remaining two groups. However, the investigation into the immunological changes resulting from *M.a. paratuberculosis* infection was confounded by concurrent coccidiosis infection.

The granulomata seen in the JPP of the "live-inoculated" lambs are similar to early paratuberculosis lesions identified by previous workers, described as "small, nodular, nonencapsulated inflammatory cell infiltrates" (Sigurdardottir *et al* 1999), "granulomata formed by cells with the appearance of macrophages" in the Peyer's patches and MLN (Perez *et al* 1996) and "well-defined but non-encapsulated" granulomata "comprised of closely-packed aggregates of macrophages with abundant cytoplasm plus fewer neutrophil polymorphs and small lymphocytes" (Begara-McGorum *et al* 1998). The early pathological changes noted in this study also bear similarities to the primary lesion of *M. bovis* infection in badgers (Gallagher *et al* 1998), described as solitary containment reactions of fibrosis and mineralisation.

AFB are not always detected in early *M.a. paratuberculosis* lesions (Nisbet *et al* 1962, Perez *et al* 1996, Begara-McGorum *et al* 1998), and were not noted in this experiment. Insufficient numbers of bacteria may be present for detection by ZN stain or they may be in an altered form. Spheroplast forms of *M.a. paratuberculosis* have been isolated from patients with Crohn's disease, and since these forms do not stain with the traditional ZN method, they cannot be visualised with this technique (Chiodini *et al* 1986, Markesich *et al* 1988, Wall *et al* 1993, McFadden and Fidler 1996). Immunohistochemistry has been suggested as a more sensitive alternative to ZN stains, since it is able to label organisms that are incomplete, or in pieces, whereas the ZN stain requires an intact cell wall (Brees *et al* 2000).

The uniformity of the FACS results between the three groups may have been due to the concurrent coccidiosis infection which masked any subtle changes due to the *M.a. paratuberculosis* infection. Coccidiosis is known to alter the levels of lymphocytes in the gut of experimentally infected lambs, especially the γδ T cells (Aleksandersen *et al* 1995). As the coccidial infection appeared to have affected the immune response in the intestines of the lambs, immunohistochemistry was not performed on the gut tissues. The coccidiosis infection prevented a meaningful comparisons to be made between the specific (group Li) and non-specific (group Hk) immune changes occurring in young animals in response to *M.a. paratuberculosis* infection.

6.4 Discussion of experiments A and B

FACS data from previous work (Begara-McGorum *et al* 1998) and experiment A (section 6.2) revealed the presence of a cmi response in young lambs inoculated with *M.a. paratuberculosis*. However, there were discrepancies regarding the lymphocyte subsets responsible for this response, summarised in Table 6-7. Begara-McGorum and workers reported higher numbers of $CD8^+$ and $CD2^+$ lymphocytes in the JPP of infected lambs, and fewer B cells in the IPP and MLN. However, while the results of experiment A concurred with the finding of lower numbers of B cells in the IPP of infected lambs, they revealed an increase in the numbers of $CD4^+$, rather than $CD8^+$ lymphocytes in the JPP, accompanied by an increase in the $CD4^+$ and a decrease in the B cells in the PBL. In contrast, no significant changes were reported in any subset of the PBL from the earlier experiment (Begara-McGorum *et al* 1998). These discrepancies may be due to the difference in design of the experiments – the original experiment (Begara-McGorum *et al* 1998) investigated a variety of time points in the first eight weeks pi, while experiment A

analysed changes occurring exactly four weeks after inoculation. Experiment A utilised more animals, and therefore had a higher statistical power, which may have allowed it to distinguish more subtle immunological changes.

Table 6-7 Comparing the changes in lymphocyte populations reported by (Begara-McGorum *et al* 1998) and shown in experiment A*

	(Begara-McGorum *et al* 1998)	Experiment A
JPP	↑$CD8^+$ ↑$CD2^+$	↑$CD4^+$
IPP	↓ B cells	↓ B cells
MLN	↓ B cells	no changes
PBL	no changes	↑$CD4^+$ ↓B cells

* The changes listed occurred in the infected groups of animals.

It is not clear why, out of a series of three related experiments, pathological and bacteriological evidence of early *M.a. paratuberculosis* infection was detected in two ((Begara-McGorum *et al* 1998) and B) but not in one (experiment A). The experimental model used in all three experiments was very similar, with the same bacterial isolate (JD88/107), detection methods, and age of lambs used. Retrospective counts were performed on the inoculum in all three experiments to ensure that the organisms used to dose the lambs were viable, and that the doses were comparable. Experiment B used a different breed of lamb – Suffolk x Texel, rather than Dorset or Suffolk x Dorset - however, it is unlikely that this would result in complete protection against *M.a. paratuberculosis* infection. The lambs in experiment B were subject to coccidiosis infection during the incubation period, which may have predisposed them to *M.a. paratuberculosis* infection, however, no evidence of coccidiosis was reported by (Begara-McGorum *et al* 1998), who successfully induced detectable *M.a. paratuberculosis* infection.

6.4.1.1 Experimental models of paratuberculosis

One of the greatest difficulties facing researchers investigating paratuberculosis is the lack of a suitable experimental model of the disease. The model described here has great potential for investigation of the *in vivo* early immune responses to mycobacterial infection. However, the high probability of various illnesses in young lambs limits the sensitive immunological data that can be collected. Young lambs are prone to a number of infections that would disrupt the

monitoring of the immunological response to *M.a. paratuberculosis* infection, including intestinal coccidiosis, respiratory disease, umbilical infections, and joint ill. Some of these diseases can be subclinical, making the interpretation of immunological responses even more difficult. The use of SPF animals would negate this problem, but increase the cost substantially, however, specific investigations into the changes in lymphocyte subsets, and levels of Th1 and Th2 cytokines in response to *M.a. paratuberculosis* infection, may require such measures. Other options for improving the model include using a different inoculation regime such as intravenous injections of *M.a. paratuberculosis*, or suppressing the immune system of the experimental animals, for example, using high doses of corticosteroids.

6.5 Conclusion

It is still not known why some animals are able to repel *M.a. paratuberculosis* infection, whereas others succumb to long term subclinical infection. Elucidation of the protective immune responses could facilitate the development of protective vaccines. This chapter reports that the early immune responses of young lambs to *M.a. paratuberculosis* infection appear to be primarily cell mediated, and further suggests a potential role for γδ T cells. Further work is needed to optimise the model used.

7 Genetic Resistance to Paratuberculosis

7.1 Introduction

There is increasing evidence that genetic factors play an important role in resistance to intracellular pathogens, including mycobacteria (discussed in section 1.12 above). Anecdotal reports suggest that the incidence of paratuberculosis is higher in some breeds of cattle and sheep (Hole and Maclay 1959, Cranwell 1993), however, no epidemiological studies investigating the incidence of paratuberculosis in different blood lines or breeds have been reported, and no polymorphisms or genetic mutations that modulate susceptibility to the disease have been identified.

The work reported in this chapter includes details of a field investigation into genetic influences on the incidence of paratuberculosis in a flock of sheep, and a molecular approach to genetic susceptibility, examining the relationship between a putative mutation in the NRAMP1 gene and the incidence of the disease.

7.2 NRAMP1 investigation

7.2.1 Hypothesis

Natural resistance associated macrophage protein (NRAMP1) is a highly conserved gene, encoding a protein known as Nramp1. NRAMP1 (allelic with Ity/Lsh/Bcg) has long been suspected of playing a vital role in susceptibility to intracellular pathogens. Nramp1 is found in the phagosome membrane of macrophages (Gruenheid *et al* 1997), and includes at least 10 hydrophobic, membrane spanning domains. Its function and mechanism of action are still unknown, although it may act as a NO_2^- or iron transporter (see section 1.12.3 above).

Vidal and workers (1993) reported a single base pair mutation in the NRAMP1 gene of certain in-bred mouse strains - BALB/cJ, and C57BL/6J. These mice exhibited increased susceptibility to several intracellular agents including BCG, *Salmonella typhimurium*, and *Leishmania donovanii* (Vidal *et al* 1993, Vidal *et al* 1995). The base pair mutation resulted in an amino acid replacement - a glycine residue was replaced by an aspartic acid residue at position 105 (Figure

7-1). This substitution occurred within one of the predicted transmembrane segments, and could be expected to alter the physical properties of this transmembrane region, and therefore the function of the protein.

This study examined the NRAMP1 gene of cattle, sheep and goats, to determine if the gly → asp mutation found in mice also occurred in ruminants (Figure 7-1), and if there was an association between the mutation and the incidence of paratuberculosis.

Figure 7-1 The base pair substitution reported in mice (Vidal *et al* 1993), resulting in a gly→asp mutation which confers increased susceptibility to intracellular pathogens, and the corresponding hypothesised mutation in ruminants.

Mouse^r	TTG	G**G**C	GGT
	leu	gly	gly
Mouse^s	TGG	G**A**C	GGT
	trp	*asp*	gly
Hypothesis:			
Ruminants^r	TGG	G**G**T	GGT
	trp	gly	gly
Ruminants^s	TGG	G**A**T	GGT
	trp	*asp*	gly

Fok 1 [GGATG(N)$_9$] can be used to differentiate between the hypothesised susceptible and resistant ruminant genotypes.

7.2.2 Materials and Methods.

7.2.2.1 Method One

Unclotted venous blood samples were collected from cattle, sheep and goats into EDTA vacutainers® (Becton Dickinson). RNA was extracted from 100µl aliquots of each sample using a DNA/RNA isolation kit (Amersham), then a 271 bp fragment of cDNA (containing the hypothesised mutation site) was amplified using the Access RT-PCR system (Promega UK). After 45 minutes at 48°C, the cycling parameters were 94°C for 2 minutes, then 40 cycles of 94°C for 30 seconds, 60°C for 1 minute and 68°C for 2 minutes and, finally, an extension phase of 68°C for 7 minutes. Primers used were primer 1: 5'GTCTGCCATCTCTACTACCC3' and primer 3: 5'TACTCATAGCCGAAGGTCAA3'.

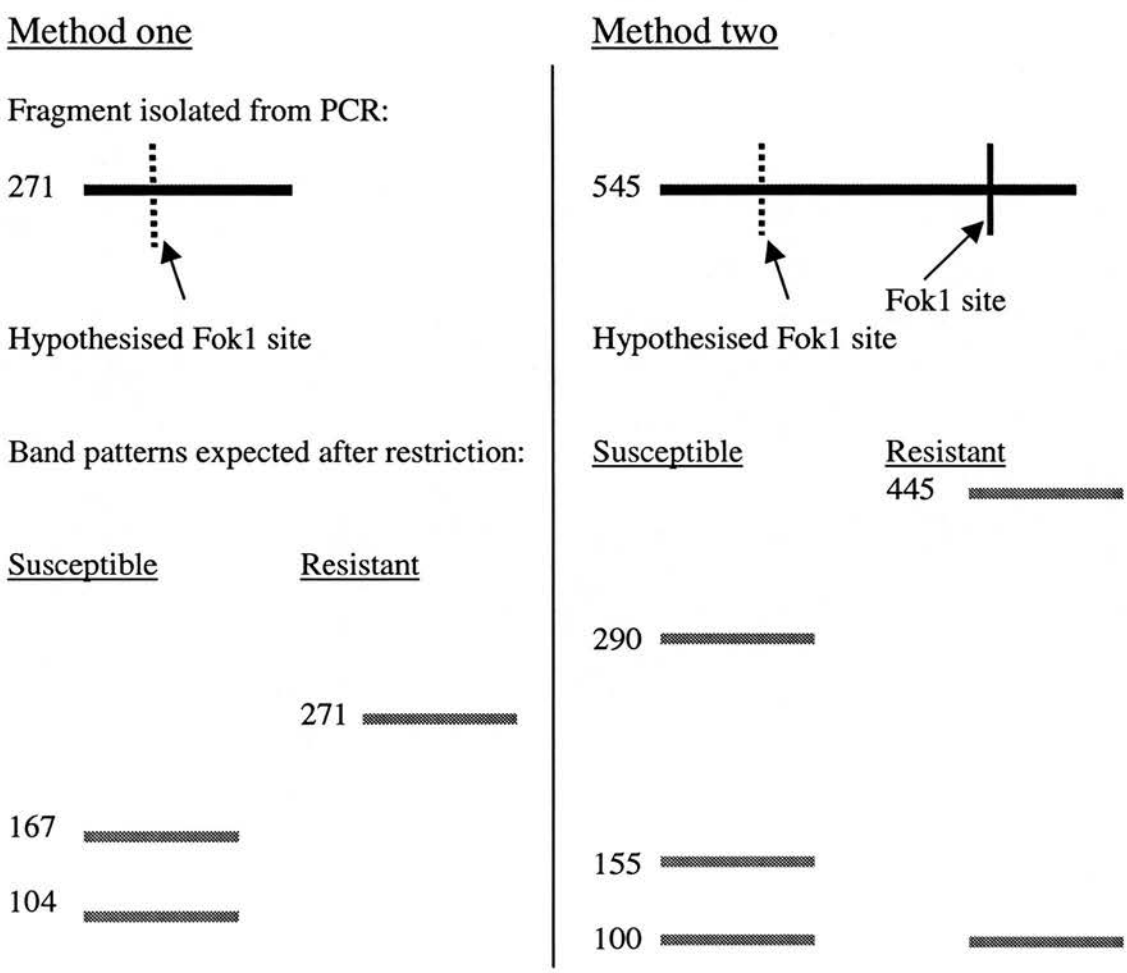
The presence of the mutation was determined by restriction with Fok1 enzyme. Briefly, 5-15µl of PCR product was added to 0.8units of Fok I (Promega UK), 2µl buffer B (6mM Tris-HCl, 6mM $MgCl_2$, 50mM NaCl, 1mM dithiothreitol, pH 7.5) (Promega UK), and 2µg acetylated BSA (Promega UK). Distilled water was added up to 20µl. The sample was then incubated at 37°C for two hours.

The PCR products from each sample were separated on a 7.5% continuous polyacrylamide gel, and visualised using a silver stain (see section 2.1.12 above). A cut in the 271 bp PCR product would indicate the presence of the mutation (Figure 7-2). 50 normal and 1 paratuberculosis affected animals were analysed using this method.

7.2.2.2 Method Two

A number of later samples were analysed with an alternative primer, primer 4: 5'ACAGCCATGACAAAGAGGTT3'. Using primer 1 and primer 4 resulted in a longer PCR fragment (545bp), including a separate Fok1 site which was used as an internal positive control for the restriction step (Figure 7-2). Five normal and two paratuberculosis affected animals were analysed using this method.

Figure 7-2 The possible Fok 1 restriction patterns from methods one and two.



7.2.2.3 Method Three

Due to the small numbers of clinical cases of paratuberculosis available, the protocol was modified to allow the use of formalin fixed, paraffin embedded (ffpe) tissues, which allowed the use of archived paratuberculosis cases. Since DNA recovery is much more reliable than RNA extraction from ffpe materials, the protocol was modified for use on ruminant NRAMP1 DNA.

From each block, a 14µm-thick section of tissue was cut on a microtome, placed in an eppendorf tube, and the paraffin wax removed using the following xylene and ethanol procedure. The section was dissolved in 1200µl of xylene, and centrifuged at 13 000 x g for 5 minutes. The xylene was removed, without disturbing the tissue pellet, and 1200µl of ethanol added. The sample was centrifuged at 13 000 x g for 5 minutes, and ethanol removed. The ethanol washing step was repeated once, and the pellet allowed to dry at 37°C for a minimum of 15 minutes. The DNA was then extracted using the QIAamp DNA extraction kit (Qiagen), eluting once in a final volume of 100µl of buffer AE.

Two samples of fresh gut - one from a case of ovine paratuberculosis, and one from a clinically normal sheep - were included as positive controls for the DNA extraction technique.

A hemi-nested PCR, using the PCR Core System I kit (Promega UK), was used to amplify a 394bp fragment of the NRAMP1 gene from the formalin fixed, paraffin wax sections. This fragment again included a separate Fok1 site 100bp from the 3′ end of the fragment, which acted as an internal positive control. Primers used were primer 8: 5′CCTAGCATGGAGGTGCCATT3′ and primer 9: 5′TCTGAGTTGCTCAAGGCCGT3′ for the first round PCR, using 10µl of template, then primer 8 again and primer 7: 5′GCGAGGTTTGCTCAGAGCCC3′ for the second round PCR adding 5µl of template from round one. The PCR cycling parameters for both rounds were 94°C for 5 minutes, followed by 40 cycles of 94°C for 1 minute, 60°C for 1 minutes, and 72°C for 2 minutes, with a final extension phase of 72°C for 5 minutes. The PCR product was restricted with Fok 1 (see section 7.2.2.1 above), and the fragments separated on a 2% 0.5X TBE agarose gel (see section 2.1.13 above) for 1.5-2hr at 70V. Two normal and 83 ruminants affected with paratuberculosis were analysed using this method.

7.2.3 Results

Method one - 50 sheep with no evidence of paratuberculosis were screened using this method, and no evidence of the hypothesised mutation was found. One clinically confirmed case of paratuberculosis was tested (caprine), and no mutation was found. This sample was also sequenced, as there was no previous report of this segment of caprine NRAMP1 cDNA. It was found to be identical to the published sheep sequence (accession number Z34916, NID g510331).

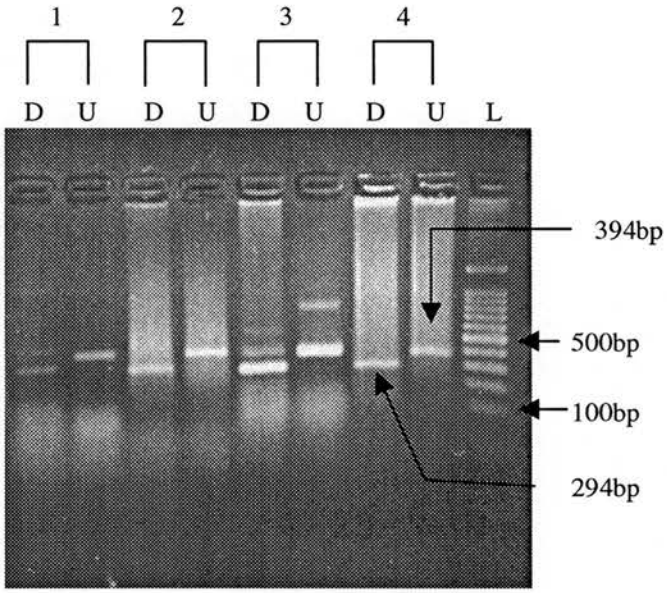
Method two - paraffin-wax embedded blocks from eight sheep with no evidence of paratuberculosis, and one cow with confirmed paratuberculosis were analysed, and no mutation found.

Method three - two samples of fresh intestines were analysed using this method, one from a sheep with paratuberculosis, and one from a clinically normal case. No mutation was found in either. Formalin fixed, paraffin-wax embedded tissues from 82 cases of paratuberculosis were analysed, but only 15 (19%) yielded sufficient DNA to restrict with the FokI enzyme (Figure 7-3). Thirteen samples were from sheep, the species of the remaining two samples was unknown. No evidence of the hypothesised mutation was found.

Combining the results from all three methods, 17 confirmed cases of paratuberculosis were screened (1 cow, 13 sheep, 1 goat, 2 species unknown), and no mutation was found. Sixty control cases were screened (1 cow, 59 sheep), and no mutation was found. Out of the 17 clinical cases of paratuberculosis that were included in the survey, 14 were classified as multibacillary and one as paucibacillary, while the histological nature of the lesions was not known for the remaining three.

The 95% confidence interval for the prevalence of the mutation in the diseased population was 0-17.6%, and the 95% confidence interval for the prevalence of the mutation in the normal population was 0-6.3%.

Figure 7-3 Restriction digests of a PCR fragment from the NRAMP gene of four paratuberculosis-affected animals, based on method three.



Fok 1 digested samples (D) are run to the left of an undigested (U) control sample. A band at 394bp can be seen in the undigested lanes, compared to 294bp following digestion, indicating that the restriction enzyme has cut at only one site, therefore the predicted gly→asp mutation is not present. L=100bp ladder, with fragment sizes as marked.

7.3 Epidemiological evidence for genetic resistance to paratuberculosis.

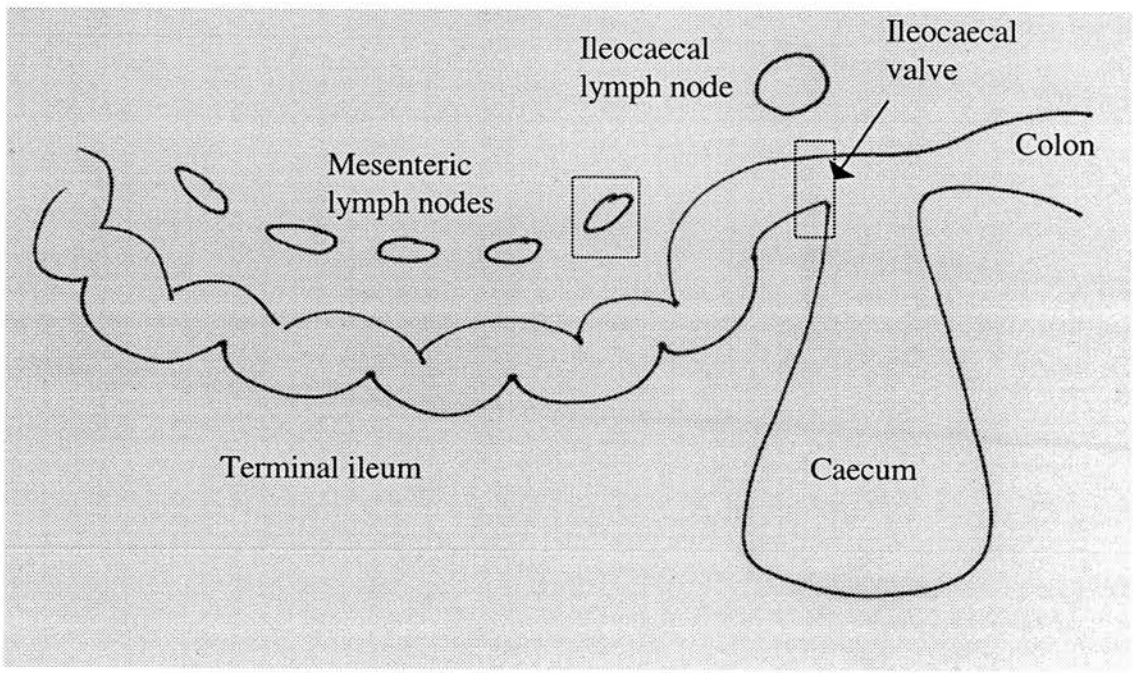
7.3.1 Hypothesis

There is anecdotal evidence of genetic susceptibility to ovine paratuberculosis in the Shetland Islands, where sheep farmers report a higher incidence of the disease in certain blood lines in their flocks (A. Clark, pers. comm.). In order to investigate these claims, a pilot study was undertaken to compare the incidence of paratuberculosis in the offspring of different sires in one flock on Shetland, to determine whether there was evidence of increased resistance to paratuberculosis in particular blood lines.

7.3.2 Materials and Methods

The flock had long term, accurate mating records, which allowed classification of animals into blood lines. The farmer reported that the offspring of a particular ram (designated ram B) seemed to be more prone than other sheep in the flock to paratuberculosis. Ram B was the only ram used in the flock during 1995 and 1996. The study analysed the paratuberculosis status of 48 cull ewes that had been born between 1990 and 1998. Forty four were examined at the Moredun Research Institute, and four by the local veterinary service. The post mortem procedure for the animals examined at the Moredun Research Institute was as follows - the animals were killed with an injection of intravenous barbiturate solution, the abdomen opened, and a sample of MLN and terminal ileum were removed (Figure 7-4), fixed in formalin for a minimum of 24 hours, then routinely processed (described in sections 2.1.5 and 2.1.6). Tissue sections were examined after staining with H&E and by the ZN method.

Figure 7-4 Diagram of the lower small intestine and caecum of a sheep, outlining the samples taken for histopathological analysis – the terminal ileum and ileocaecal valve.



Using the flock records of the farm, the prevalence of paratuberculosis in the offspring of ram B was compared to the prevalence in the offspring of other rams used on the farm in the past 10 years.

7.3.3 Results

Examination at the Moredun Research Institute of 27 ewes sired by ram B found histological evidence suggestive of *M.a. paratuberculosis* infection in 11. Farm records showed that a further two had been diagnosed with paratuberculosis - one by histopathology, and one by clinical signs and a positive ZN faecal smear. Therefore 13 out of 29 animals examined that were sired by ram B showed evidence of paratuberculosis infection (Table 7-1). Examination of offspring from other rams used on the farm between 1990 and 1998 showed only two out of 19 had lesions consistent with a diagnosis of paratuberculosis.

Table 7-1 A comparison of the number of infected animals in the offspring of ram B with the number of infected offspring sired by other rams on the farm.

	number of animals positive*	total number examined
offspring of ram B	13	29
offspring of other rams	2	19

*Positive by histopathological examination (H&E and ZN stained sections)

Analysis of the two ratios (ram B offspring = 13 out of 29, and others = 2 out of 19), showed a statistically significant difference, p=0.019, using Fisher's Exact Test.

7.4 Discussion

Preliminary results from an infected sheep flock in Shetland (study 7.3) reveal that the host genotype has a significant effect on susceptibility to paratuberculosis, but PCR analysis of the genome of 77 animals (study 7.2) suggests that the NRAMP mutation which results in decreased resistance to intracellular pathogens in mice is unlikely to occur in ruminants.

7.4.1 Role of NRAMP1 in susceptibility to paratuberculosis

The gly→asp mutation identified in the NRAMP1 gene of mice is unlikely to occur at a prevalence of greater than 6.3% in the normal ruminant population, and 17.6% in the paratuberculosis-affected ruminant population. No statement can be made about the relationship between the mutation and disease, given that no mutation was found.

7.4.2 Comparison of methods used in study 7.2

Analysis of DNA from fresh tissue (either blood or post mortem tissue) in study 7.2 was quicker and more productive than DNA from ffpe tissue. All samples from fresh tissue, compared to only 19% of ffpe samples, yielded sufficient DNA for analysis. However, DNA from ffpe tissues can be used to analyse a larger population of animals with paratuberculosis, since archived material can be utilised. Routine histological sections can also be cut and stained from the ffpe block, allowing histological details of the case to be assessed.

A low number of ffpe cases (19%) yielded sufficient DNA to allow PCR of the NRAMP1 fragment. Factors affecting ffpe PCR include the length of the amplified fragment, the length of time the tissue was fixed in formalin, age of the sample, and the number of copies of target DNA (Benezra *et al* 1991, Shibata 1994). The length of time in formalin was unknown for the majority of samples, some samples were up to six years old, and the length of amplified product was relatively long at 394bp. These factors would have decreased the sensitivity of the technique and could explain the low success rate when using ffpe tissues.

7.4.3 Epidemiological evidence of susceptibility to paratuberculosis

The results of study 7.3 revealed that the progeny of ram B have a statistically significantly higher risk of developing paratuberculosis when compared to offspring of other rams from the same farm. However, there were difficulties in separating the influences of environmental and genetic factors on the study population. The offspring of ram B were the only lambs born on the farm during 1996 and 1997. It is possible that environmental contamination with *M.a. paratuberculosis* may have been particularly high during these years, or that the animals were subjected to increased stress which in turn increased the risk of paratuberculosis.

Conventional control measures for a number of important infectious diseases, especially of sheep, are becoming increasingly ineffective and uneconomic. Consequently, there has recently been an emphasis on developing strategies for genetic approaches to control diseases such as parasitic gastroenteritis, flystrike and footrot. The aim of such work is to allow farmers to breed flocks with increased disease resistance. A full review of such programmes, which is beyond the scope of this thesis, has recently been published (Raadsma *et al* 1998). The results from the pilot study described in this chapter into the heritability of susceptibility to paratuberculosis suggest that such programmes may be suitable for inclusion into control programmes for paratuberculosis, especially ovine paratuberculosis, where economic constraints restrict the current control options available to producers.

Such control programmes would require a means of assessing the resistance of animals to paratuberculosis. Under ideal circumstances, this would be possible without first exposing the animal to the pathogen. The most effective means of doing this is by identifying DNA markers for resistance, and then genotyping the animals at the marker loci in order to determine their

resistance or susceptibility. This approach is used in the scrapie genotype testing currently being carried out in a number of British sheep breeds.

The NRAMP1 gene has been linked to susceptibility to other mycobacterial diseases and is therefore a potential DNA marker for paratuberculosis. In the study described in this chapter, no evidence of a specific gly→asp mutation was found, however, there are further polymorphisms and mutations in the gene that could be investigated such as those described in cattle (Horin *et al* 1999) or humans (Liu *et al* 1995). Genetic susceptibility to mycobacterial diseases has also been associated with MHC haplotypes (Todd *et al* 1990, Kubo *et al* 2000), the locus *sst-1* (Kramnik *et al* 2000), vitamin D receptor gene polymorphisms (Bellamy *et al* 1999, Gelder *et al* 2000), and IL-12 and IFN-γ receptor mutations (Altare *et al* 1998, Jouanguy *et al* 1999b), promoting these genes as possible candidates for providing suitable DNA markers for paratuberculosis.

7.5 Conclusion

Control of paratuberculosis in infected flocks and herds currently depends on a test and cull strategy, combined with suitable management practices and vaccination. The results presented in this chapter suggest that that it may be worthwhile attempting to identify susceptible and resistant genotypes which in turn may allow selection of livestock with increased resistance to paratuberculosis. This would greatly enhance existing control programmes.

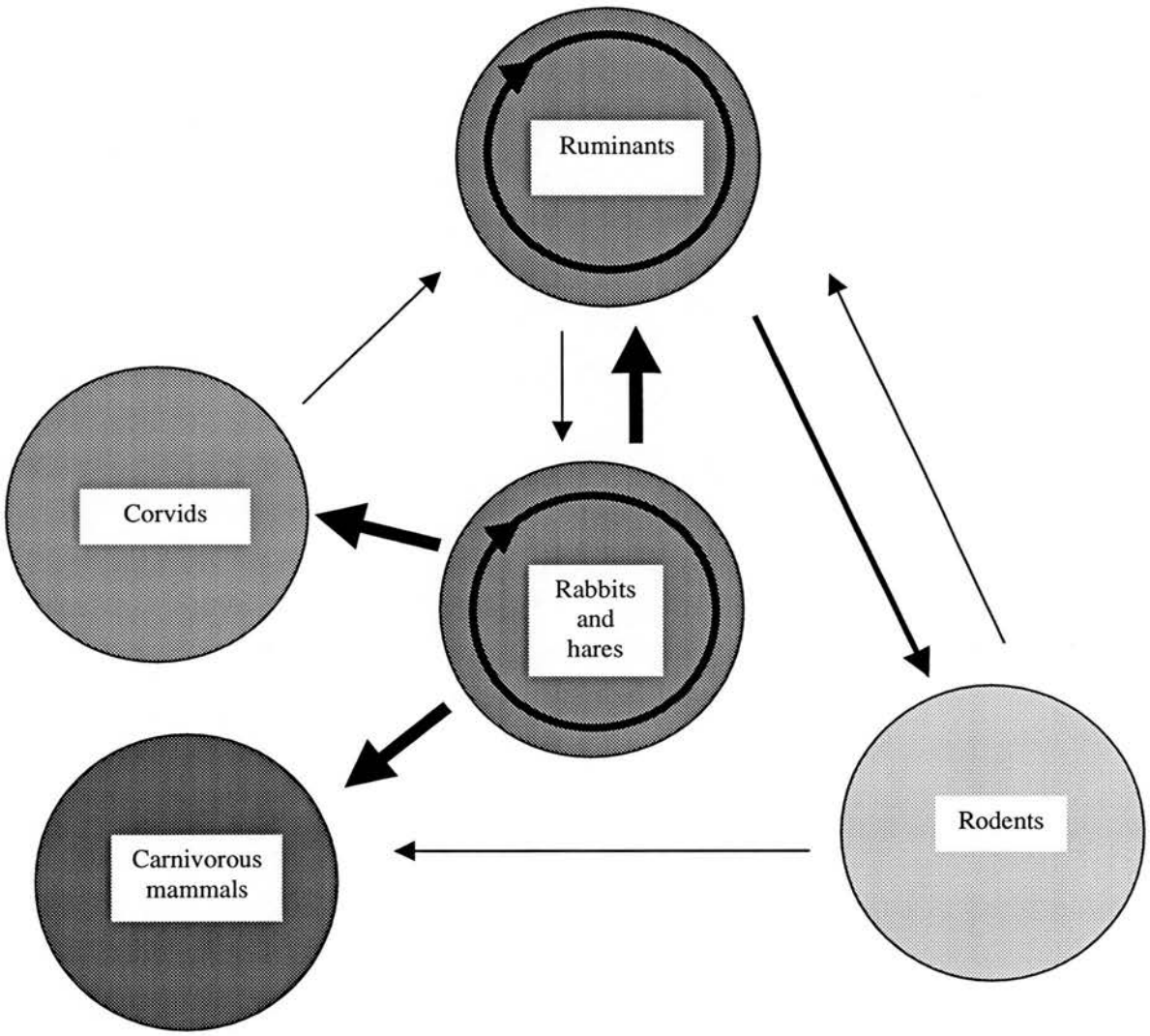
8 General Discussion

The investigations described in this thesis into the epidemiology and pathogenesis of paratuberculosis involved studies into non-ruminant sylvatic cycles of *M.a. paratuberculosis*, the changes associated with initial *M.a. paratuberculosis* infection in young ruminants, and genetic susceptibility to paratuberculosis.

The results from a pilot study described in chapter 7 reveal a significant influence of sire on the probability of a sheep having paratuberculosis-like lesions evident on histopathological examination. While this suggests a significant influence of host genotype on the susceptibility and resistance to paratuberculosis, the study was based on a limited sample size, and confounding environmental factors could not be disregarded. If supported by further, more extensive investigations, this finding may be utilised in disease control programmes similar to those already established to combat footrot and parasitic gastroenteritis in sheep (Raadsma *et al* 1998). Further investigations may incorporate research for DNA markers linked to resistance to paratuberculosis which would facilitate selection of resistant animals, as well as provide information on the pathogenesis of the disease. Examples of this have been recorded in human tuberculosis, where mutations in the IL-12 gene resulted in increased susceptibility to *M. tuberculosis* (Jouanguy *et al* 1999a), thereby revealing the importance of this cytokine in the immune response against the pathogen.

Investigation of non-ruminant wildlife from paratuberculosis-affected farms in the east of Scotland revealed a wide range of animals to be infected with *M.a. paratuberculosis,* especially carnivorous and scavenging species. Evidence of infection was found in foxes, stoats, weasels, rabbits, crows, rooks, jackdaws, rats, wood mice, hares and badgers, suggesting that the epidemiology of paratuberculosis is more complex than was previously realised. Studies of the pathology and pathogenesis of the disease in the various wildlife species, as well as investigations into potential interspecies transmission routes, have suggested a link between these wildlife reservoirs of *M.a. paratuberculosis* and the disease in ruminants (Figure 8-1).

Figure 8-1 Diagram of the suggested interactions between the cycles of *M.a. paratuberculosis* in different groups of animals.



The species involved have been classified as either herbivourous grazers (rabbits and hares), carnivorous mammals (foxes, stoats and weasels), carrion-eating corvids (crows, rooks and jackdaws), or rodents (rats and mice). The size of the arrows represents the extent to which it is suggested that the organism is transmitted between the groups of animals.

Most information has been gathered on the cycle of *M.a. paratuberculosis* in rabbits. There is growing evidence supporting the theory of transmission of *M.a. paratuberculosis* between rabbits and cattle. Previous work identified a statistical link between rabbit and ruminant paratuberculosis, and failed to identify a species-specific strain of the organism (Greig *et al* 1999), both facts supporting the hypothesis that interspecies transmission does occur. Experimental infections have since shown that a *M.a. paratuberculosis* isolate from a free-living rabbit is pathogenic for calves, producing lesions consistent with a diagnosis of paratuberculosis (see section 5.2 above). In studies of potential transmission routes, it was found that rabbits excrete significant numbers of *M.a. paratuberculosis* in their faeces, while cattle do not avoid grazing pasture contaminated with rabbit faeces (M. J. Daniels, submitted). As the *M.a. paratuberculosis* organism is known to survive for months on pasture, rabbit faeces therefore represent a very real risk for ruminant *M.a. paratuberculosis* infection.

While the route and potential impact of transmission of *M.a. paratuberculosis* from rabbits to cattle is becoming clearer, the method and extent of cattle to rabbit transmission is still unknown. It is feasible that the factors which increase the amount of *M.a. paratuberculosis* on pasture would increase the risk of rabbits catching the disease. A high prevalence of paratuberculosis in the ruminant livestock on the farm, and intensive management practices resulting in extensive faecal contamination of pastures and low sward heights may provide an environment conducive to interspecies transmission of paratuberculosis. A large population of rabbits would increase the grazing pressure on the pasture, and therefore increase the risk that they would ingest sufficient *M.a. paratuberculosis* from the cattle faeces on the pasture to cause disease.

The means by which *M.a. paratuberculosis* passes between rabbits is also incompletely understood. The coprophagic habits of rabbits suggest that ingestion of doe faeces by her kits may be a possible transmission route, while the recovery of *M.a. paratuberculosis* from the urine of severely affected rabbits implies that *M.a. paratuberculosis* infection is widespread in these animals, possibly extending to the mammary tissues. The organism may therefore be excreted in the milk of a lactating doe, representing a potential source of infection for nursing kits.

However, experimental work has suggested that neonatal rabbits are resistant to oral inoculation with *M.a. paratuberculosis* (see section 5.3 above). Studies are continuing into this finding as

the reason for the apparent resistance may be due to the breed or age of rabbit used in the experiments, as discussed earlier. If, however, free-living rabbits are genuinally resistant to oral inoculation with *M.a. paratuberculosis*, cattle to rabbit spread of the organism may be a rare occurrence. Once the organism is in the rabbit population, however, it may be readily transmitted within the species, possibly vertically, with infected rabbits then representing a risk to both ruminants and carnivores.

The difficulties encountered in reproducing rabbit paratuberculosis experimentally and the unique pathological features described in affected rabbits suggest that the pathogenesis of the disease may differ from that in ruminants. Indeed, considering the physiology of the rabbit digestive system, it is perhaps no surprise that the disease should be different. Rabbits are hind gut fermenters, and practice coprophagy, which may enhance the cycling of the *M.a. paratuberculosis* organism, and assist in the formation of the florid lesions described in chapter 3. Further investigations to clarify the pathogenesis of rabbit paratuberculosis, including defining the relative susceptibilities of rabbits of different ages, the presence or absence of a subclinical phase of the disease and the extent of lesions in severely affected rabbits, may allow us to understand how and why the disease occurs, reveal the most likely routes of inter and intraspecies transmission, and, if necessary, take appropriate action to prevent transmission to farmed ruminants and carnivorous wildlife.

While a number of carnivorous species have been found to be infected with *M.a. paratuberculosis*, there is no evidence that they develop clinical disease. The lesions identified in the fox, stoat, weasel and crow have been subtle, with few organisms identified. It is probable that these species are dead end hosts of the organism, unable to perpetuate *M.a. paratuberculosis* infection without contact with infected rabbits, and play little part in the epidemiology of the disease. However, *M.a. paratuberculosis* has been recovered from the faeces of a number of carnivorous species, so they are a potential risk for domestic ruminants. Birds especially have the ability to contaminate feedstuffs and also to disperse the organism over considerable distances.

Rodents are infected at low levels (rats at 9% and mice at 3%), and pathological evidence of infection is rare and mild. Consequently, it is unlikely that these species play an important role in the epidemiology of paratuberculosis, although they do have the potential to contaminate

ruminant feedstuffs, as well as act as prey and therefore a source of infection for carnivorous mammals.

It is unclear why Tayside is a "hotspot" of wildlife paratuberculosis, since the factors influencing the creation and maintenance of a sylvatic cycle of *M.a. paratuberculosis* in a wildlife population remain to be elucidated. It was shown that 22% of free-living rabbits and 89% of foxes in Tayside were infected with *M.a. paratuberculosis*, however, significantly fewer rabbits in other areas of Scotland and Northern England were found to have evidence of the disease. This could be due to the level of paratuberculosis in the farmed ruminants, or particular farm management practices as discussed earlier. Alternatively, despite the failure to find evidence of a species-specific strain, it is still possible that *M.a. paratuberculosis* in wildlife is the result of a mutated strain of the organism present in Tayside area, which has increased virulence for rabbits and possibly also for other wildlife. Further study of farms with and without *M.a. paratuberculosis*-infected wildlife may result in a better understanding of the causes of paratuberculosis in free-living populations.

The impact of concurrent infection on the susceptibility of animals to paratuberculosis has not been widely investigated. However, opportunistic infections during the course of this work have provided evidence to suggest that enteric coccidiosis infection may enhance the virulence of *M.a. paratuberculosis*. Experimental infection of SPF rabbits was unsuccessful (chapter 5), in contrast to the evidence of paratuberculosis in both experimental (Mokresh *et al* 1989, Mokresh and Butler 1990) and free-living (chapter 3) populations of coccidiosis-infected rabbits. Lambs also developed lesions consistent with *M.a. paratuberculosis* infection only when suffering from a concurrent coccidiosis infection (chapter 6). Finally, experimental infection of calves with *M.a. paratuberculosis* was successful, again with a background of incidental enteric coccidiosis. The rabbit, lamb and calf experiments were not designed to investigate the role of concurrent disease in determining the susceptibility of animals to infection with *M.a. paratuberculosis*, however, the results suggest that this hypothesis deserves further research.

Investigations into the immunopathogenesis of early paratuberculosis in ruminants have revealed other factors which may influence the susceptibility of animals to *M.a. paratuberculosis* infection. Cell mediated immunity appears to be the predominant initial host response to *M.a. paratuberculosis* inoculation, in line with results from investigations into other mycobacterial

diseases. It may be that animals capable of mounting a quick and effective cmi response, with high levels of Th1 cytokines, are able to resist infection whereas *M.a. paratuberculosis* can take advantage of a "sluggish" response in other animals, reaching the relative safety of the cytoplasm of the macrophage in the GALT.

As well as a rise in cmi, *M.a. paratuberculosis* inoculation is associated with a significant and previously unreported increase in the numbers of γδ T cells in the GALT of the intestine. The role of these γδ T cells is unclear - they may help the host, the pathogen, or neither. It is known that γδ T cells have the ability to lyse *M. tuberculosis*-infected macrophages, and release anti-mycobacterial substances (Dieli *et al* 2000), consequently, γδ T cells may play an important role in early defence by killing the *M.a. paratuberculosis* organisms that cross the intestinal mucosa into the underlying Peyer's patches. Alternatively, γδ T cells have been implicated in granuloma formation in murine *M.a. paratuberculosis* infection (Tanaka *et al* 2000). Granulomata provide *M.a. paratuberculosis* organisms with a suitable niche for long term survival, thus γδ T cells may in fact assist the pathogen to avoid the host's defence mechanisms. This argument may be used to explain the increased susceptibility of young ruminants to *M.a. paratuberculosis* infection, as the number of γδ T cells, and therefore the host's ability to form granulomata, declines with age. There remains the possibility that γδ T cells play an insignificant part in the pathogenesis of paratuberculosis, and that the rise in γδ T cells in the GALT of inoculated calves is a non-specific response to foreign material in the gut lumen.

The inconsistent results from the immunopathological experiments described in chapter 6 highlight the incomplete understanding we have of the events occurring when the animal is first exposed to *M.a. paratuberculosis*. In the experiment described in section 6.2, 30 lambs were each inoculated with more than $5x10^7$ organisms, but no bacteriological or pathological evidence of infection was detected in any. However, *M.a. paratuberculosis* infection was detected in lambs from similar experiments (Begara-McGorum *et al* 1998)(section 6.3), while previous reports described successful infection of lambs with an oral dose containing as few as $1x10^3$ organisms (Brotherston *et al* 1961a). The discrepancies between experimental results suggest that the infective dose of *M.a. paratuberculosis* is influenced by a number of factors, including host genotype, the presence of concurrent infection such as intestinal coccidiosis, and even age. It has been reported that young animals are more susceptible to *M.a. paratuberculosis* infection,

however, the 30 lambs described in section 6.2 were less than three weeks old, yet all exhibited remarkable resistance to inoculation with live *M.a. paratuberculosis*. Clarification of the factors influencing susceptibility to *M.a. paratuberculosis* infection will lead to a better understanding of the pathogenesis of the disease, improvements in the experimental models of paratuberculosis, and, hopefully, better disease control methods.

Knowledge of the existence and impact that wildlife reservoirs of *M.a. paratuberculosis* have on the disease in domestic ruminants may allow the implementation of more successful on-farm control programmes, elucidation of the earliest events that occur in *M.a. paratuberculosis* infection may result in the development of more effective vaccines, and a better understanding of the role of host genotype in the susceptibility to paratuberculosis may be utilised to select animals with above average resistance to the disease. A more accurate and extensive knowledge of the epidemiology and pathogenesis of paratuberculosis will therefore facilitate the control and prevention of this significant disease of livestock.

References

Abbas AK, Lichtman AH, Pober JS (1994) *Cellular and Molecular Immunology*, 2nd edition, W.B. Saunders

Abel L, Sanchez FO, Oberti J, Thuc NV, VanHoa L, Lap VD, Skamene E, Lagrange PH, Schurr E (1998) Susceptibility to leprosy is linked to the human NRAMP1 gene, *Journal of Infectious Diseases* **177,** 133-145

Abrahamsen MS (1998) Bovine T cell responses to *Cryptosporidium parvum* infection, *International Journal for Parasitology* **28,** 1083-1088

Abrahamsen MS, Lancto CA, Walcheck B, Layton W, Jutila MA (1997) Localization of alpha/beta and gamma/delta T lymphocytes in *Cryptosporidium parvum*-infected tissues in naive and immune calves, *Infection and Immunity* **65,** 2428-2433

Ackermann MR, Cheville NF, Deyoe BL (1988) Bovine ileal dome lymphoepithelial cells: endocytosis and transport of *Brucella abortus* strain 19, *Veterinary Pathology* **25,** 28-35

Agranoff D, Monahan IM, Mangan JA, Butcher PD, Krishna S (1999) *Mycobacterium tuberculosis* expresses a novel pH-dependent divalent cation transporter belonging to the Nramp family, *Journal of Experimental Medicine* **190,** 717-724

Akol GW, Authie E, Pinder M, Moloo SK, Roelants GE, Murray M (1986) Susceptibility and immune responses of zebu and taurine cattle of West Africa to infection with *Trypanosoma congolense* transmitted by *Glossina morsitans centralis*, *Veterinary Immunology and Immunopathology* **11,** 361-373

Aleksandersen M, Landsverk T, Gjerde B, Helle O (1995) Scarcity of gamma-delta T-cells in intestinal epithelia containing coccidia despite general increase of epithelial lymphocytes, *Veterinary Pathology* **32,** 504-512

Allen JE, Maizel RM (1997) Th1-Th2: reliable paradigm or dangerous dogma?, *Immunology Today* **18,** 387-392

Allworth, B (2000) National Ovine Johne's Disease Control and Evaluation Program Surveillance Report, **3** Animal Health Australia

Almeria S, Canals A, Zarlenga DS, Gasbarre LC (1997) Isolation and phenotypic characterization of abomasal mucosal lymphocytes in the course of a primary *Ostertagia ostertagi* infection in calves, *Veterinary Immunology and Immunopathology* **57,** 87-98

Altare F, Jouanguy E, Lamhamedi S, Doffinger R, Fischer A, Casanova JL (1998) Mendelian susceptibility to mycobacterial infection in man, *Current Opinion In Immunology* **10,** 413-417

Alzuherri HM, Woodall CJ, Clarke CJ (1996) Increased intestinal TNF-alpha, IL-1 beta and IL-6 expression in ovine paratuberculosis, *Veterinary Immunology and Immunopathology* **49,** 331-345

Angus KW (1990) Intestinal lesions resembling paratuberculosis in a wild rabbit (*Oryctolagus cuniculus*), *Journal of Comparative Pathology* **103,** 101-105

Angus KW, Gilmour NJL (1971) Effect of the rimino phenazine B663 (G30320) on *Mycobacterium johnei* infection and reinfection in sheep II Pathology, *Journal of Comparative Pathology* **81,** 227-234

Anon. (1980) Invermay Animal Health Laboratory report: Tuberculosis in rabbits, *Surveillance* **7,** 22-23

Anon. (1999) Wildlife diseases in Europe, Ed. European Wildlife Disease Association, Annual report to OIE,

Anon. (2000) Scottish Agricultural College Veterinary Science Division summary, *Veterinary Record* **146,** 5

Armitage P, Berry G (1994) *Statistical Methods in Medical Research*, 3rd edition, Blackwell Science

Augustin A, Kubo RT, Sim GK (1989) Resident pulmonary lymphocytes expressing the gamma-delta T-cell receptor, *Nature* **340,** 239-241

Baldwin CL, Sathiyaseelan T, Rocchi M, McKeever DJ (2000) Rapid changes occur in the percentage of circulating bovine WC1(+)gammadelta Th1 cells, *Research in Veterinary Science* **69,** 175-180

Barker IK, van Dreumel AA, Palmer N (1993) The alimentary system, in *Pathology of Domestic Animals*, 4th edition, ed. Jubb KVP, Kennedy PC, and Palmer N, Academic Press, San Diego p 1-318

Barnes PF, Abrams JS, Lu S, Sieling PA, Rea TH, Modlin RL (1993a) Patterns of cytokine production by mycobacterium-reactive human T-cell clones, *Infection and Immunity* **61,** 197-203

Barnes PF, Lu S, Abrams JS, Wang E, Yamamura M, Modlin RL (1993b) Cytokine production at the site of disease in human tuberculosis, *Infection and Immunity* **61,** 3482-3489

Barnes PF, Wizel B (2000) Type 1 cytokines and the pathogenesis of tuberculosis, *American Journal of Respiratory and Critical Care Medicine* **161,** 1773-1774

Barthel R, Piedrahita JA, McMurray DN, Payeur J, Baca D, Suarez GF, Perumaalla VS, Ficht TA, Templeton JW, Adams LG (2000) Pathologic findings and association of *Mycobacterium bovis* infection with the bovine NRAMP1 gene in cattle from herds with naturally occurring tuberculosis, *American Journal of Veterinary Research* **61,** 1140-1144

Bassey EOE, Collins MT (1997) Study of T-lymphocyte subsets of healthy and *Mycobacterium avium* subsp. *paratuberculosis*-infected cattle, *Infection and Immunity* **65,** 4869-4872

Beckman EM, Melian A, Behar SM, Sieling PA, Chatterjee D, Furlong ST, Matsumoto R, Rosat JP, Modlin RL, Porcelli SA (1996) CD1c restricts responses of mycobacteria-specific T cells. Evidence for antigen presentation by a second member of the human CD1 family, *Journal of Immunology* **157,** 2795-2803

Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB (1994) Recognition of a lipid antigen by CD1-restricted alpha-beta(+) T-cells, *Nature* **372,** 691-694

Begara-McGorum I, Wildblood LA, Clarke CJ, Connor KM, Stevenson K, McInnes CJ, Sharp JM, Jones DG (1998) Early immunopathological events in experimental ovine paratuberculosis, *Veterinary Immunology and Immunopathology* **63,** 265-287

BehrPerst SI, Munk ME, Schaberg T, Ulrichs T, Schulz RJ, Kaufmann SHE (1999) Phenotypically activated gamma delta T lymphocytes in the peripheral blood of patients with tuberculosis, *Journal of Infectious Diseases* **180,** 141-149

Bellamy R (1999) The natural resistance-associated macrophage protein and susceptibility to intracellular pathogens, *Microbes and Infection* **1,** 23-27

Bellamy R, Hill AVS (1998) Genetic susceptibility to mycobacteria and other infectious pathogens in humans, *Current Opinion In Immunology* **10,** 483-487

Bellamy R, Ruwende C, Corrah T, McAdam KP, Thursz M, Whittle HC, Hill AV (1999) Tuberculosis and chronic hepatitis B virus infection in Africans and variation in the vitamin D receptor gene, *Journal of Infectious Diseases* **179,** 721-724

Bellamy R, Ruwende C, Corrah T, McAdam KPWJ, Whittle HC, Hill AVS (1998) Variations in the Nramp1 gene and susceptibility to tuberculosis in West Africans, *New England Journal of Medicine* **338,** 640-644

Bendixen PH (1978) Immunological reactions caused by infection with *Mycobacterium paratuberculosis*, *Nordic Veterinary Medicine* **19,** 163-168

Bendixen PH, Bloch B, Jorgensen J (1981) Lack of intracellular degregation of *Mycobacterium paratuberculosis* by bovine macrophages infected in vitro and in vivo: light microscopic and electron microscopic observations, *American Journal of Veterinary Research* **42,** 109-113

Benedictus G, Dijkhuizen AA, Stelwagen J (1987) Economic losses due to paratuberculosis in dairy cattle, *Veterinary Record* **121,** 142-146

Benezra J, Johnson DA, Rossi J, Cook N, Wu A (1991) Effect of fixation on the amplification of nucleic-acids from paraffin-embedded material by the polymerase chain-reaction, *Journal of Histochemistry and Cytochemistry* **39,** 351-354

Bertotto A, Gerli R, Spinozzi F, Muscat C, Scalise F, Castellucci G, Sposito M, Candio F, Vaccaro R (1993) Lymphocytes bearing the gamma-delta T-cell receptor in acute *Brucella melitensis* infection, *European Journal of Immunology* **23,** 1177-1180

Bird P, Jones P, Allen D, Donachie W, Huntley J, Mcconnell I, Hopkins J (1995) Analysis of the expression and secretion of isotypes of sheep B cell immunoglobulins with a panel of isotype-specific monoclonal antibodies, *Research in Veterinary Science* **59,** 189-194

Boom WH (1999) Gamma delta T cells and *Mycobacterium tuberculosis*, *Microbes and Infection* **1,** 187-195

Born W, Happ MP, Dallas A, Reardon C, Kubo R, Shinnick T, Brennan P, Obrien R (1990) Recognition of heat-shock proteins and gamma-delta cell function, *Immunology Today* **11,** 40-43

Born W, Obrien R (1990) The gamma-delta cell response to stress - unresolved issues and possible significance, *Research in Immunology* **141,** 595-600

Bossers A, Belt PBGM, Raymond GJ, Caughey B, deVries R, Smits MN (1997) Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms, *Proceedings of the National Academy of Sciences of the United States of America* **94,** 4931-4936

Brees DJ, Reimer SB, Cheville NF, Florance A, Thoen CO (2000) Immunohistochemical detection of *Mycobacterium paratuberculosis* in formalin-fixed, paraffin-embedded bovine tissue sections, *Journal of Veterinary Diagnostic Investigation* **12,** 60-63

Brennan PJ, Nikaido H (1995) The envelope of mycobacteria, *Annual Review of Biochemistry* **64,** 29-63

Brenner MB, Mclean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, Seidman JG, Ip S, Rosen F, Krangel MS (1986) Identification of a putative second T-cell receptor, *Nature* **322,** 145-149

Briones V, de Juan L, Sanchez C, Vela AI, Galka M, Montero, Goyache J, Aranaz A, Dominguez L (2000) Bovine tuberculosis and the endangered Iberian lynx, *Emerging Infectious Diseases* **6,** 189-191

Brooke WS (1941) The vole acid fast bacillus, *American Review of Tuberculosis* **43,** 806-816

Brotherston JG, Gilmour NJL, McSamuel J (1961b) Quantitative studies of *Mycobacterium johnei* in the tissues of sheep II Protection afforded by dead vaccines, *Journal of Comparative Pathology* **71,** 300-310

Brotherston JG, Gilmour NJL, Samuel JM (1961a) Quantitative Studies of *Mycobacterium johnei* in the tissues of sheep I Routes of infection and assay of viable *M. johnei*, *Journal of Comparative Pathology* **71,** 286-299

Brown H, Prescott R. Applied mixed models in medicine. 1999. Chichester, John Wiley.

BruningFann CS, Schmitt SM, Fitzgerald SD, Payeur JB, Whipple DL, Cooley TM, Carlson T, Friedrich P (1998) *Mycobacterium bovis* in coyotes from Michigan, *Journal of Wildlife Diseases* **34,** 632-636

Bruschke CJM, Haghparast A, Hoek A, Rutten VPMG, Wentink GH, vanRijn PA, vanOirschot JT (1998) The immune response of cattle, persistently infected with noncytopathic BVDV, after superinfection with antigenically semi-homologous cytopathic BVDV, *Veterinary Immunology and Immunopathology* **62,** 37-50

Buddle BM, Young LJ (2000) Immunobiology of mycobacterial infections in marsupials, *Developmental and Comparative Immunology* **24,** 517-529

Buergelt CD, Ginn P (1999) The histopathological diagnosis of early Johne's disease in North American bison (*Bison bison*), in *Proceedings of the Sixth International Colloquium on Paratuberculosis, February 14-18 1999, Melbourne.*, ed. Manning EJB and Collins MT, International Association for Paratuberculosis, Madison, WI p 395-399

Buergelt CD, Hall C, McEntree K, Duncan JR (1978) Pathological evaluation of paratuberculosis in naturally infected cattle, *Veterinary Pathology* **15,** 196-207

Bull T, Pavlik I, Hermontaylor J, ElZaatari F, Tizard M (1999) Study of IS900 loci in *Mycobacterium avium* subsp *paratuberculosis* by multiplex PCR screening, in *Proceedings of the Sixth International Colloquium on Paratuberculosis, February 14-18 1999, Melbourne.*, ed. Manning EJB and Collins MT, International Association for Paratuberculosis, Madison, WI p 265-274

Burdin N, Kronenberg M (1999) CD1-mediated immune responses to glycolipids, *Current Opinion In Immunology* **11,** 326-331

Burrells C, Clarke CJ, Colston A, Kay JM, Porter J, Little D, Sharp JM (1998) A study of immunological responses of sheep clinically-affected with paratuberculosis (Johne's disease) - The relationship of blood, mesenteric lymph node and intestinal lymphocyte responses to gross and microscopic pathology, *Veterinary Immunology and Immunopathology* **66,** 343-358

Burrells C, Clarke CJ, Colston A, Kay JM, Porter J, Little D, Sharp JM (1999) Interferon-gamma and interleukin-2 release by lymphocytes derived from the blood, mesenteric lymph nodes and intestines of

normal sheep and those affected with paratuberculosis (Johne's disease), *Veterinary Immunology and Immunopathology* **68,** 139-148

Burrells C, Inglis NF, Davies RC, Sharp JM (1995) Detection of specific T-cell reactivity in sheep infected with *Mycobacterium avium* subspecies *silvaticum* and *paratuberculosis* using 2 defined mycobacterial antigens, *Veterinary Immunology and Immunopathology* **45,** 311-320

Burrells C, Palmarini G, Porter J, Stevenson K, Sharp JM (1996) Experimental infection of red deer with *Mycobacterium avium* subspecies *paratuberculosis* and IS901$^+$ *Mycobacterium avium*, in *Proceedings of the Fifth International Colloquium on Paratuberculosis*, ed. Chiodini RJ, Hines ME, and Collins MT, International Association for Paratuberculosis, Madison p 196-201

Buxton D (2000) Toxoplasmosis and neosporosis, in *Diseases of Sheep*, 3 edition, ed. Martin WB and Aitken ID, Blackwell Science Ltd, p 86-94

Buxton D, Reid HW, Finlayson J, Pow I (1984) Pathogenesis of sheep-associated malignant catarrhal fever in rabbits, *Research in Veterinary Science* 205-211

Calabi F, Jarvis JM, Martin L, Milstein C (1989) Two classes of CD1 genes, *European Journal of Immunology* **19,** 285-292

Caley P (1998) Broad-scale possum and ferret correlates of macroscopic *Mycobacterium bovis* infection in feral ferret populations, *New Zealand Veterinary Journal* **46,** 157-162

CanonneHergaux F, Gruenheid S, Govoni G, Gros P (1999) The Nramp1 protein and its role in resistance to infection and macrophage function, *Proceedings Of The Association Of American Physicians* **111,** 283-289

Carrigan MJ, Seaman JT (1990) The pathology of Johnes disease in sheep, *Australian Veterinary Journal* **67,** 47-50

Cetinkaya B, Egan K, Harbour DA, Morgan KL (1996) An abattoir-based study of the prevalence of subclinical Johne's disease in adult cattle in south west England, *Epidemiology and Infection* **116,** 373-379

Cetinkaya B, Erdogan HM, Morgan KL (1998) Prevalence, incidence and geographical distribution of Johne's disease in cattle in England and the Welsh borders, *Veterinary Record* **143,** 265-269

Chaitaweesub P, Abbott KA, Whittington R, Marshall DJ (1999) Shedding of organisms and subclinical effects on production on preclinical merino sheep affected with paratuberculosis, in *Proceedings of the Sixth International Colloquium on Paratuberculosis, February 14-18 1999, Melbourne.*, ed. Manning EJB and Collins MT, International Association for Paratuberculosis, Madison, WI p 126-131

Challans JA, Stevenson K, Reid HW, Sharp JM (1994) A rapid method for the extraction and detection of *Mycobacterium avium* subspecies *paratuberculosis* from clinical specimens, *Veterinary Record* **134,** 95-96

Chalmers RM, Sturdee AP, Bull SA, Miller A, Wright SE (1997) The prevalence of *Cryptosporidium parvum* and *C. muris* in *Mus domesticus, Apodemus sylvaticus* and *Clethrionomys glareolus* in an agricultural system, *Parasitological Research* **83,** 478-482

Chiba T, Hiraga M, Aida Y, Ajito T, Asahina M, Wu D, Ohshima K, Davis WC, Okada K (1995) Immunohistologic studies on subpopulations of lymphocytes in cattle with enzootic bovine leukosis, *Veterinary Pathology* **32,** 513-520

Chiodini RJ (1989) Crohn's disease and the mycobacterioses - a review and comparison of 2 disease entities, *Clinical Microbiology Reviews* **2,** 90-117

Chiodini RJ, Davis WC (1992) The cellular immunology of bovine paratuberculosis - the predominant response is mediated by cytotoxic gamma delta T-lymphocytes which prevent CD4+ activity, *Microbial Pathogenesis* **13,** 447-463

Chiodini RJ, Davis WC (1993) The cellular immunology of bovine paratuberculosis - immunity may be regulated by CD4+ helper and CD8+ immunoregulatory T-lymphocytes which down regulate gamma delta T cell cytotoxicity, *Microbial Pathogenesis* **14,** 355-367

Chiodini RJ, Van Kruiningen HJ, Thayer WR, Coutu JA (1986) Spheroplastic phase of mycobacteria isolated from patients with Crohn's disease, *Journal of Clinical Microbiology* **24,** 357-363

Chiodini RJ, Vankruiningen HJ (1983) Eastern white-tailed deer as a reservoir of ruminant paratuberculosis, *Journal of the American Veterinary Medical Association* **182,** 168-169

Chiodini RJ, Vankruiningen HJ, Merkal RS (1984) Ruminant paratuberculosis (Johne's disease) - the current status and future prospects, *Cornell Veterinarian* **74,** 218-262

Choy E, Whittington RJ, Marsh I, Marshall J, Campbell MT (1998) A method for purification and characterisation of *Mycobacterium avium* subsp. *paratuberculosis* from the intestinal mucosa of sheep with Johne's disease, *Veterinary Microbiology* **64,** 51-60

Clarke CJ (1997) The pathology and pathogenesis of paratuberculosis in ruminants and other species, *Journal of Comparative Pathology* **116,** 217-261

Clarke CJ, Little D (1996) The pathology of ovine paratuberculosis: Gross and histological changes in the intestine and other tissues, *Journal of Comparative Pathology* **114,** 419-437

Clarke CJ, Patterson IAP, Armstrong KE, Low JC (1996) Comparison of the absorbed ELISA and agar gel immunodiffusion test with clinicopathological finding in ovine clinical paratuberculosis, *Veterinary Record* **139,** 618-621

Coetsier C, Havaux X, Mattelard F, Sadatte S, Cormont F, Buergelt K, Limbourg B, Latinne D, Bazin H, Denef JF, Cocito C (1998) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in infected tissues by new species-specific immunohistological procedures, *Clinical and Diagnostic Laboratory Immunology* **5,** 446-451

Collins DM, Gabric DM, Delisle GW (1990) Identification of 2 groups of *Mycobacterium paratuberculosis* strains by restriction endonuclease analysis and DNA hybridization, *Journal of Clinical Microbiology* **28,** 1591-1596

Collins MT (1996) Diagnosis of paratuberculosis, *Veterinary Clinics of North America-Food Animal Practice* **12,** 357-371

Collins P, Davies DC, Matthews PRJ (1984) Mycobacterial infection in goats - diagnosis and pathogenicity of the organism, *British Veterinary Journal* **140,** 196-201

Converse PJ, Dannenberg AM, Estep JE, Sugisaki K, Abe Y, Schofield BH, Pitt MLM (1996) Cavitary tuberculosis produced in rabbits by aerosolized virulent tubercle bacilli, *Infection and Immunity* **64,** 4776-4787

Converse PJ, Dannenberg AM, Shigenaga T, McMurray DN, Phalen SW, Stanford JL, Rook GAW, KoruSengul T, Abbey H, Estep JE, Pitt MLM (1998) Pulmonary bovine-type tuberculosis in rabbits: Bacillary virulence, inhaled dose effects, tuberculin sensitivity, and *Mycobacterium vaccae* immunotherapy, *Clinical and Diagnostic Laboratory Immunology* **5,** 871-881

Cooke MM, Jackson R, Coleman JD (1993) Tuberculosis in a free-living brown hare (*Lepus europaeus occidentalis*), *New Zealand Veterinary Journal* **41,** 144-146

Cooper AM, Flynn JL (1995) The protective immune response to *Mycobacterium tuberculosis*, *Current Opinion In Immunology* **7,** 512-516

Corpa JM, Garrido J, Garcia Marin JF, Perez V (2000) Classification of lesions observed in natural cases of paratuberculosis in goats, *Journal of Comparative Pathology* **122,** 255-265

Cousins D, Whittington RJ, Masters A, Marsh I, Evans R, Kluver P (1999) Investigation of false positives in the IS900 PCR for identification of *Mycobacterium avium* subsp. *paratuberculosis*, in *Proceedings of the Sixth International Colloquium on Paratuberculosis, February 14-18 1999, Melbourne*, ed. Manning EJB and Collins MT, International Association for Paratuberculosis, Madison, WI p 259-264

Covert TC, Rodgers MR, Reyes AL, Stelma GN (1999) Occurrence of nontuberculous mycobacteria in environmental samples, *Applied And Environmental Microbiology* **65,** 2492-2496

Cranwell MP (1993) Control of Johne's disease in a flock of sheep by vaccination, *Veterinary Record* **133,** 219-220

Crocker G, Sopp P, Parsons K, Davis WC, Howard CJ (1993) Analysis of the gamma delta T-cell restricted antigen WC1, *Veterinary Immunology and Immunopathology* **39,** 137-144

Cross ML, Chinn ND, Griffin JFT, Buchan GS (1996) T cell responses to *Mycobacterium bovis* in red deer, a large animal model for tuberculosis, *Immunology and Cell Biology* **74,** 32-37

Dannenberg AM, Jr. (1991) Delayed-type hypersensitivity and cell-mediated immunity in the pathogenesis of tuberculosis, *Immunology Today* **12,** 228-233

Dargatz DA, Wells SJ (1999) Johne's disease on US beef operations: results from the NAHMS Beef 97 study, in *Proceedings of the Sixth International Colloquium on Paratuberculosis*, ed. Manning EJB and Collins MT, International Association for Paratuberculosis, Madison, WI p 59-61

Daubenberger CA, Taracha ELN, Gaidulis L, Davis WC, McKeever DJ (1999) Bovine gamma delta T-cell responses to the intracellular protozoan parasite *Theileria parva*, *Infection and Immunity* **67,** 2241-2249

de Chastellier C, Lang T, Thilo L (1995) Phagocytic processing of the macrophage endoparasite, *Mycobacterium avium*, in comparison to phagosomes which contain *Bacillus subtilis* or latex beads, *European Journal of Cell Biology* **68,** 167-182

Dieli F, Troye-Blomberg M, Ivanyi J, Fournie JJ, Bonneville M, Peyrat MA, Sireci G, Salerno A (2000) Vgamma9/Vdelta2 T lymphocytes reduce the viability of intracellular *Mycobacterium tuberculosis*, *European Journal of Immunology* **30,** 1512-1519

Doyle TM (1953) Susceptibility to Johne's disease in relation to age, *Veterinary Record* **65,** 363-365

Doyle TM (1954) Isolation of Johne's bacilli from the udder of clinically affected cows, *British Veterinary Journal* **110,** 215-218

Doyle TM (1958) Foetal infection in Johne's disease, *Veterinary Record* **70,** 238

Doyle TM (1960) Vaccination against Johne's disease, *British Veterinary Journal* **116,** 294-301

Ellingson JLE, Stabel JR, Whitlock RH (1999) Detection of *M.a. paratuberculosis* in free-ranging bison by PCR, in *Proceedings of the Sixth International Colloquium on Paratuberculosis*, ed. Manning EJB and Collins MT, International Association for Paratuberculosis, Madison, WI p 420-423

Ellis TM, Carson BA, Kalkhoven MJ, Martin PAJ (1998) Specificity of two absorbed ELISAs for surveys of *Mycobacterium paratuberculosis* in cattle, *Australian Veterinary Journal* **76,** 497-499

Emoto M, Danbara H, Yoshikai Y (1992) Induction of gamma delta T cells in murine salmonellosis by an avirulent but not by a virulent strain of *Salmonella choleraesuis*, *Journal of Experimental Medicine* **176,** 363-372

Escayg AP, Hickford JGH, Bullock DW (1997) Association between alleles of the ovine major histocompatibility complex and resistance to footrot, *Research in Veterinary Science* **63,** 283-287

Evans CW, Lund BT, Mcconnell I, Bujdoso R (1994) Antigen recognition and activation of ovine gamma delta T cells, *Immunology* **82,** 229-237

Falkinham JO, III (1996) Epidemiology of infection by nontuberculous mycobacteria, *Clinical Microbiology Reviews* **9,** 177-215

Faure F, Jitsukawa S, Miossec C, Hercend T (1990) CD1C as a target recognition structure for human T-lymphocytes. Analysis with peripheral blood gammadelta cells, *European Journal of Immunology* **20,** 703-706

Feizabadi MM, Robertson ID, Hope A, Cousins DV, Hampson DJ (1997) Differentiation of Australian isolates of *Mycobacterium paratuberculosis* using pulsed field gel electrophoresis, *Australian Veterinary Journal* **75,** 887-889

Fenhalls G, Wong A, Bezuidenhout J, van Helden P, Bardin P, Lukey PT (2000) In situ production of gamma interferon, interleukin-4, and tumor necrosis factor alpha mRNA in human lung tuberculous granulomas, *Infection and Immunity* **68,** 2827-2836

Fine PEM (1981) Immunogenetics of susceptibility to leprosy, tuberculosis, and leishmaniasis. An epidemiological perspective, *International Journal of Leprosy* **49,** 437-454

Fodstad FH, Gunnarsson E (1979) Post-mortem examination of in the diagnosis of Johne's disease in goats, *Acta Veterinaria Scandinavica* **20,** 157-167

Forbes TDA, Hodgson J (1985) The reaction of grazing sheep and cattle to the presence of dung from the same or the other species, *Grass and Forage Science* **40,** 177-182

Francis J (1943) Infection of laboratory animals with *Mycobacterium johnei*, *Journal of Comparative Pathology and Theriogenology* **53,** 140-150

Frelier PF, Templeton JW, Estes M, Whitford HW, Kienle RD (1990) Genetic regulation of *Mycobacterium paratuberculosis* infection in recombinant inbred mice, *Veterinary Pathology* **27,** 362-364

Fridriksdottir V, Gunnarsson E, Sigurdarson S, Gudmundsdottir KB (1999) Paratuberculosis in Iceland: Epidemiology and Control Measures, Past and Present, in *Proceedings of the Sixth International Colloquium on Paratuberculosis, February 14-18 1999, Melbourne.*, ed. Manning EJB and Collins MT, International Association for Paratuberculosis, Madison, WI p 105-108

Fujimura Y, Owen RL (1996) M cells as portals of infection: clinical and pathophysiological aspects, *Infectious Agents and Disease* **5,** 144-156

Gallagher J, Monies R, GavierWiden M, Rule B (1998) Role of infected, non-diseased badgers in the pathogenesis of tuberculosis in the badger, *Veterinary Record* **142,** 710-714

Garcia Marin JF, Tellechea J, Gutierrez M, Perez V, Juste RA (1997) Paratuberculosis vaccination in sheep modifies and limits the development of lesions, in *Proceedings of the Fifth International Colloquium on Paratuberculosis, September 29-October 4 1996*, ed. Chiodini R, Hines ME, and Collins MT, International Association for Paratuberculosis, Madison, WI p 223

Gelder CM, Hart KW, Williams OM, Lyons E, Welsh KI, Campbell IA, Marshall SE (2000) Vitamin D receptor gene polymorphisms and susceptibility to *Mycobacterium malmoense* pulmonary disease, *Journal of Infectious Diseases* **181,** 2099-2102

Gilmour NJL, Angus KW (1971) Effect of rimino phenazine B663 (G30320) on *Mycobacterium johnei* infection and reinfection in sheep I Bacteriology and hypersensitivity, *Journal of Comparative Pathology* **81,** 221-226

Gilmour NJL, Angus KW, Mitchell B (1978) Intestinal infection and host response to oral administration of *Mycobacterium johnei* in sheep, *Veterinary Microbiology* **2,** 223-235

Gilmour NJL, Nyange J (1989) Paratuberculosis (Johne's disease) in deer, *In Practice* 193-196

Gomes MS, Appelberg R (1998) Evidence for a link between iron metabolism and Nramp1 gene function in innate resistance against *Mycobacterium avium*, *Immunology* **95,** 165-168

Goodger WJ, Collins MT, Nordlund KV, Eisele C, Pelletier J, Thomas CB, Sockett DC (1996) Epidemiologic study of on-farm management practices associated with prevalence of *Mycobacterium paratuberculosis* infections in dairy cattle, *Journal of American Veterinary Medical Association* **208,** 1877-1881

Grant IR, Ball HJ, Rowe MT (1998) Isolation of *Mycobacterium paratuberculosis* from milk by immunomagnetic separation, *Applied And Environmental Microbiology* **64,** 3153-3158

Green EP, Tizard ML, Moss MT, Thompson J, Winterbourne DJ, McFadden JJ, Hermon-Taylor J (1989) Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*, *Nucleic Acids Research* **17,** 9063-9073

Greig A (2000) Johne's disease in sheep and goats, *In Practice* **22,** 146-151

Greig A, Stevenson K, Henderson D, Perez V, Hughes V, Pavlik I, Hines ME, McKendrick I, Sharp JM (1999) Epidemiological study of paratuberculosis in wild rabbits in Scotland, *Journal of Clinical Microbiology* **37,** 1746-1751

Greig A, Stevenson K, Perez V, Pirie AA, Grant JM, Sharp JM (1997) Paratuberculosis in wild rabbits (*Oryctolagus cuniculus*), *Veterinary Record* **140,** 141-143

Gruenheid S, Pinner E, Desjardins M, Gros P (1997) Natural resistance to infection with intracellular pathogens: The Nramp1 protein is recruited to the membrane of the phagosome, *Journal of Experimental Medicine* **185,** 717-730

Gunnarsson E (1979) Isolation of *Mycobacterium paratuberculosis* from sheep and cattle in Iceland, *Acta Veterinaria Scandinavica* **20,** 191-199

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter, *Nature* **388,** 482-488

Gwozdz JM, Reichel MP, Murray A, Manktelow W, West DM, Thompson KG (1997) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in ovine tissues and blood by the polymerase chain reaction, *Veterinary Microbiology* **57,** 233-244

Hagan WA (1938) Age as a factor in susceptibility to Johne's disease, *Cornell Veterinarian* **28,** 34-40

Hallman ET, Witter JF (1933) Some observations on the pathology of Johne's disease, *Journal of American Veterinary Medical Association* **83,** 159-187

Harding H (1959) The histopathology of *Mycobacterium johnei* infection in small laboratory animals, *Journal of Pathology and Bacteriology* **78,** 157-169

Haregewoin A, Singh B, Gupta RS, Finberg RW (1991) A mycobacterial heat-shock protein-responsive gamma-delta T cell clone also responds to the homologous human heat-shock protein - a possible link between infection and autoimmunity, *Journal of Infectious Diseases* **163,** 156-160

Havran WL, Chien YH, Allison JP (1991) Recognition of self antigens by skin-derived T cells with invariant gamma delta antigen receptors, *Science* **252,** 1430-1432

Hein WR, Dudler L (1997) TCR gamma delta(+) cells are prominent in normal bovine skin and express a diverse repertoire of antigen receptors, *Immunology* **91,** 58-64

Hein WR, Mackay CR (1991) Prominence of gamma-delta T-cells in the ruminant immune system, *Immunology Today* **12,** 30-34

Hietala SK (1992) The options in diagnosing ruminant paratuberculosis, *Veterinary Medicine* **87,** 1122-1132

Himes EM, Miller S, Miller LD, Jarnagin JL (1989) *Mycobacterium avium* isolated from a domestic rabbit with lesions in the central nervous system, *Journal of Veterinary Diagnostic Investigation* **1,** 76-78

Hines ME, Frazier K (1993) Differentiation of mycobacteria on the basis of chemotype profiles by using matrix solid-phase dispersion and thin-layer chromatography, *Journal of Clinical Microbiology* **31,** 610-614

Hines SA, Buergelt CD, Wilson JH, Bliss EL (1987) Disseminated *Mycobacterium paratuberculosis* infection in a cow, *Journal of the American Veterinary Medical Association* **190,** 681-683

Hirch A (1956) Infection of hamsters and rabbits with *Mycobacterium johnei*, *Journal of Comparative Pathology* **66,** 260-269

Ho M, Tongtawe P, Kriangkum J, Wimonwattrawatee T, Pattanapanyasat K, Bryant L, Shafiq J, Suntharsamai P, Looareesuwan S, Webster HK, Elliott JF (1994) Polyclonal expansion of peripheral gamma-delta T cells in human *Plasmodium falciparum* malaria, *Infection and Immunity* **62,** 855-862

Hoad, G. (2000) Preliminary results from the National Study on the microbiological quality and heat processing of cows milk: *Mycobacterium avium* subsp *paratuberculosis*, **ACM/485** p 2-6 Advisory Committee on the Microbiological Safety of Food, www.foodstandards.gov.uk

Hoft DF, Brown RM, Roodman ST (1998) Bacille Calmette-Guerin vaccination enhances human gamma delta T cell responsiveness to mycobacteria suggestive of a memory-like phenotype, *Journal of Immunology* **161,** 1045-1054

Hole NH, Maclay MH (1959) 1. The diagnosis of Johne's disease in cattle and the identification of *M johnei* infection, *Veterinary Record* **71,** 1145-1149

Hopkins J, Dutia BM, Rhind SM (2000) Sheep CD1 genes and proteins, *Veterinary Immunology and Immunopathology* **73,** 3-14

Horin P, Rychlik I, Templeton JW, Adams LG (1999) A complex pattern of microsatellite polymorphism within the bovine NRAMP1 gene, *European Journal of Immunogenetics* **26,** 311-313

Howard AD, Zwilling BS (1999) Reactivation of tuberculosis is associated with a shift from type 1 to type 2 cytokines, *Clinical and Experimental Immunology* **115,** 428-434

Howard AD, Zwilling BS (1998) Cytokine production by CD4 and CD8 T cells during the growth of *Mycobacterium tuberculosis* in mice, *Clinical and Experimental Immunology* **113,** 443-449

Howard CJ, Sopp P, Bembridge G, Young J, Parsons KR (1993) Comparison of CD1 monoclonal antibodies on bovine cells and tissues, *Veterinary Immunology and Immunopathology* **39,** 77-83

Huang JH, Oefner PJ, Adi V, Ratnam K, Ruoss SJ, Trako E, Kao PN (1998) Analyses of the NRAMP1 and IFN-gamma R1 genes in women with *Mycobacterium avium-intracellulare* pulmonary disease, *American Journal of Respiratory and Critical Care Medicine* **157,** 377-381

Hubbard J, Surawicz CM (1999) Etiological role of mycobacterium in Crohn's disease - An assessment of the literature, *Digestive Diseases* **17,** 6-13

Hughes MS, James G, Ball N, Scally M, Malik R, Wigney DI, Martin P, Chen S, Mitchell D, Love DN (2000) Identification by 16S rRNA gene analyses of a potential novel mycobacterial species as an etiological agent of canine leproid granuloma syndrome, *Journal of Clinical Microbiology* **38,** 953-959

Hutchings MR, Harris S (1999) Quantifying the risks of TB infection to cattle posed by badger excreta, *Epidemiology and Infection* **122,** 167-173

Hutchings MR, Harris S (1997) Effects of farm management practices on cattle grazing behaviour and the potential for transmission of bovine tuberculosis from badgers to cattle, *Veterinary Journal* **153,** 149-162

Inglis, N. F. (1997) Studies on a 40kDa protein antigen of IS901/902-positive *Mycobacterium avium*, PhD Thesis, University of Edinburgh

Inoue T, Yoshikai Y, Matsuzaki G, Nomoto K (1991) Early appearing gamma-delta bearing T-cells during infection with Calmette-Guerin bacillus, *Journal of Immunology* **146,** 2754-2762

Janis EM, Kaufmann SHE, Schwartz RH, Pardoll DM (1989) Activation of gamma-delta T-cells in the primary immune response to *Mycobacterium tuberculosis*, *Science* **244,** 713-716

Jansen J (1948) Paratuberculosis, *Journal of American Veterinary Medical Association* **112,** 52-54

Jessup DA, Abbas B, Behymer D, Gogan P (1981) Paratuberculosis in tule elk in California, *Journal of the American Veterinary Medical Association* **179,** 1252-1254

Johne HA, Frothingham L (1895) Ein eigenthuemlicher Fall von Tuberkulose beim Rind, *Duetsche Zeitschrift fur Tiermedicin und Pathologie* **21,** 438-454

Johnson-Ifearulundu Y, Kaneene JB (1999) Distribution and environmental risk factors for paratuberculosis in dairy cattle herds in Michigan, *American Journal of Veterinary Research* **60,** 589-596

Jolly PE, Gangopadhyay A, Chen SD, Reddy PG, Weiss HL, Sapp WJ (1997) Changes in the leukocyte phenotype profile of goats infected with the caprine arthritis encephalitis virus, *Veterinary Immunology and Immunopathology* **56,** 97-106

Jones DG, Kay JM (1996) Serum biochemistry and the diagnosis of Johne's disease (paratuberculosis) in sheep, *Veterinary Record* **139,** 498-499

Jones TC, Hunt RD, King NW (1997) Mineral deposits and pigments, in *Veterinary Pathology*, 6 edition, ed. Jones TC, Hunt RD, and King NW, Williams and Wilkins, p 57-81

Jorgensen JB (1977) Survivial of *Mycobacterium paratuberculosis* in slurry, *Nordic Veterinary Medicine* **29,** 267-270

Jouanguy E, Doffinger R, Dupuis S, Pallier A, Altare F, Casanova JL (1999a) IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men, *Current Opinion In Immunology* **11,** 346-351

Jouanguy E, LamhamediCherradi S, Lammas D, Dorman SE, Fondaneche MC, Dupuis S, Doffinger R, Altare F, Girdlestone J, Emile JF, Ducoulombier H, Edgar D, Clarke J, Oxelius VA, Brai M, Novelli V, Heyne K, Fischer A, Holland SM, Kumararatne DS, Schreiber RD, Casanova JL (1999b) A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection, *Nature Genetics* **21,** 370-378

JouenBeades F, Paris E, Dieulois C, Lemeland JF, BarreDezelus V, Marret S, Humbert G, Leroy J, Tron F (1997) In vivo and in vitro activation and expansion of gamma delta T cells during *Listeria monocytogenes* infection in humans, *Infection and Immunity* **65,** 4267-4272

Jubb, Kennedy, Palmer (1991) Infectious and Parasitic Diseases of the Gastrointestinal tract, in *Pathology of Domestic Animals*, 4 edition, ed. Jubb, Kennedy, and Palmer, Academic Press, San Diego p 247-252

Juste RA, Marin JFG, Peris B, Deocariz CS, Badiola JJ (1994) Experimental infection of vaccinated and non-vaccinated lambs with *Mycobacterium paratuberculosis*, *Journal of Comparative Pathology* **110,** 185-194

Kabelitz D, Wesch D, Hinz T (1999) Gamma delta T cells, their T cell receptor usage and role in human diseases, *Springer Seminars In Immunopathology* **21,** 55-75

Kalis CHJ, Hesselink JW, Russchen EW, Barkema HW, Collins MT, Visser IJR (1999) Factors influencing the isolation of *Mycobacterium avium* subsp *paratuberculosis* from bovine fecal samples, *Journal of Veterinary Diagnostic Investigation* **11,** 345-351

Kao RR, Roberts MG (1999) A comparison of wildlife control and cattle vaccination as methods for the control of bovine tuberculosis, *Epidemiology and Infection* **122,** 505-519

Kaufmann SHE (1996) Gamma/delta and other unconventional T lymphocytes: What do they see and what do they do?, *Proceedings of the National Academy of Sciences of the United States of America* **93,** 2272-2279

King CM (1991) Stoat *Mustela erminea*, in *A Handbook of British Mammals*, 3 edition, ed. Corbet GB and Harris S, Blackwell Scientific Press, Oxford p 351-387

Kluge JP, Merkal RS, Monlux WS, Larsen AB, Kopecky KE, Ramsey FK, Lehmann RP (1968) Experimental paratuberculosis in sheep after oral, intratrachael, or intravenous inoculation: lesions and demonstration of etiologic agent, *American Journal of Veterinary Research* **29,** 953-962

Koenig GJ, Hoffsis GF, Shulaw WP, Bechnielsen S, Rings DM, Stjean G (1993) Isolation of *Mycobacterium paratuberculosis* from mononuclear cells in tissues, blood, and mammary glands of cows with advanced paratuberculosis, *American Journal of Veterinary Research* **54,** 1441-1445

Koets, A. P. (2000) Patterns of T-cell responses in different stages of bovine paratuberculosis, PhD Thesis, Utrecht University, The Netherlands

Koets AP, Rutten VPMG, Hoek A, Bakker D, vanZijderveld F, Muller KE, vanEden W (1999) Heat-shock protein-specific T-cell responses in various stages of bovine paratuberculosis, *Veterinary Immunology and Immunopathology* **70,** 105-115

Kopecky KE, Larsen AB, Merkal RS (1967) Uterine infection in bovine paratuberculosis, *American Journal of Veterinary Research* **28,** 1043-1045

Koshiba M, Ogawa K, Hamazaki S, Sugiyama T, Ogawa O, Kitajima T (1993) The effect of formalin fixation on DNA and the extraction of high- molecular-weight DNA from fixed and embedded tissues, *Pathology Research and Practice* **189,** 66-72

Kramnik I, Dietrich WF, Demant P, Bloom BR (2000) Genetic control of resistance to experimental infection with virulent *Mycobacterium tuberculosis*, *Proceedings of the National Academy of Sciences of the United States of America* **97,** 8560-8565

Krebs, J. R. (1997) Bovine tuberculosis in cattle and badgers, Ministry of Agriculture, London

Kreeger JM (1991) Ruminant paratuberculosis - a century of progress and frustration, *Journal of Veterinary Diagnostic Investigation* **3,** 373-383

Kruuk H, Parish T (1982) Factors affecting population density, group size and territory size of the European badger *Meles meles*, *Journal of Zoology, London* **196,** 31-39

Kubo K, Yamazaki Y, Hanaoka M, Nomura H, Fujimoto K, Honda T, Ota M, Kamijou Y (2000) Analysis of HLA antigens in *Mycobacterium avium-intracellulare* pulmonary infection, *American Journal of Respiratory and Critical Care Medicine* **161,** 1368-1371

Lang F, Peyrat MA, Constant P, Davodeau F, David-Ameline J, Poquet Y, Vie H, Fournie JJ, Bonneville M (1995) Early activation of human V gamma 9V delta 2 T cell broad cytotoxicity and TNF production by nonpeptidic mycobacterial ligands, *Journal of Immunology* **154,** 5986-5994

Lang T, Prina E, Sibthorpe D, Blackwell JM (1997) Nramp1 transfection transfers Ity/Lsh/Bcg-related pleiotropic effects on macrophage activation: Influence on antigen processing and presentation, *Infection and Immunity* **65,** 380-386

Larsen AB, Kopecky KE (1970) *Mycobacterium paratuberculosis* in reproductive organs and semen of bulls, *American Journal of Veterinary Research* **31,** 255-257

Larsen AB, Merkal RS, Cutlip RC (1975) Age of cattle as related to resistance to infection with *Mycobacterium paratuberculosis*, *American Journal of Veterinary Research* **36,** 255-257

Larsen AB, Merkal RS, Vardaman T (1956) Survival time of *Mycobacterium paratuberculosis*, *American Journal of Veterinary Research* **17,** 549-551

Larsen AB, Miller JM (1972) Mammary gland exposure of cows to *Mycobacterium paratuberculosis*, *American Journal of Veterinary Research* **39,** 1972-1974

Larsen AB, Miller JM (1979) Susceptibility of the Lemming (*Dicrostonyx rubricatus*) to *Mycobacterium paratuberculosis*, *American Journal of Veterinary Research* **40,** 1657-1658

Leckie FM, Thirgood SJ, May R, Redpath SM (1998) Variation in the diet of red foxes on Scottish moorland in relation to prey abundance, *Ecography* **21,** 599-604

Leighton FA, Artois M, Capucci L, Gavierwiden D, Morisse JP (1995) Antibody response to rabbit viral hemorrhagic disease virus in red foxes (*Vulpes vulpes*) consuming livers of infected rabbits (*Oryctolagus cuniculus*), *Journal of Wildlife Diseases* **31,** 541-544

Levi ML (1948) Experimental study of Johne's disease in goats, *Journal of Comparative Pathology* **58,** 38-63

Lillini E, Gamberale F, DiGuardo G (1999) *Mycobacterium avium* subsp *paratuberculosis* infection in a water buffalo (*Bubalus bubalis*) from Central Italy, in *Proceedings of the Sixth International Colloquium on Paratuberculosis, February 14-18 1999, Melbourne.*, ed. Manning EJB and Collins MT, International Association for Paratuberculosis, Madison, WI p 254

Linklater KA (2000) Other infectious cases of abortion, in *Diseases of Sheep*, 3rd edition, ed. Martin WB and Aitken ID, Blackwell Science Ltd, p 107-113

Little, D. (1997) The pathology and immunology of paratuberculosis in sheep and goats, PhD Thesis, University of Edinburgh

Little D, Alzuherri HM, Clarke CJ (1996) Phenotypic characterisation of intestinal lymphocytes in ovine paratuberculosis by immunohistochemistry, *Veterinary Immunology and Immunopathology* **55,** 175-187

Liu J, Fujiwara TM, Buu NT, Sanchez FO, Cellier M, Paradis AJ, Frappier D, Skamene E, Gros P, Morgan K, Schurr E (1995) Identification of polymorphisms and sequence variants in the human homolog of the mouse natural resistance-associated macrophage protein gene, *American Journal of Human Genetics* **56,** 845-853

Lovell R, Levi.M., Francis J (1944) Studies on the survival of Johne's bacilli, *Journal of Comparative Pathology* **54,** 120-129

Lucas J, Lucas A, Furber H, James G, Hughes MS, Martin P, Chen SCA, Mitchell DH, Love DN, Malik R (2000) *Mycobacterium genavense* infection in two aged ferrets with conjunctival lesions, *Australian Veterinary Journal* **78,** 685-689

Lugton IW (1999) Mucosa-associated lymphoid tissues as sites for uptake, carriage and excretion of tubercle bacilli and other pathogenic mycobacteria, *Immunology and Cell Biology* **77,** 364-372

Lugton IW, Wobeser G, Morris RS, Caley P (1997) Epidemiology of *Mycobacterium bovis* infection in feral ferrets (*Mustela furo*) in New Zealand.1. Pathology and diagnosis, *New Zealand Veterinary Journal* **45,** 140-150

Machugh ND, Mburu JK, Carol MJ, Wyatt CR, Orden JA, Davies WC (1997) Identification of two distinct subsets of bovine gamma delta T cells with unique cell surface phenotype and tissue distribution, *Immunology* **92,** 340-345

Mackay CR, Hein WR, Brown MH, Matzinger P (1988) Unusual expression of CD2 in sheep: implications for T cell interactions, *European Journal of Immunology* **18,** 1681-1688

Mackintosh CG, Qureshi T, Waldrup K, Labes RE, Dodds KG, Griffin JF (2000) Genetic resistance to experimental infection with *Mycobacterium bovis* in red deer (*Cervus elaphus*), *Infection and Immunity* **68,** 1620-1625

Malik R, Love DN, Wigney DI, Martin P (1998) Mycobacterial nodular granulomas affecting the subcutis and skin of dogs (canine leproid granuloma syndrome), *Australian Veterinary Journal* 403-407

Marcato P (1974) Histopathology of lymph nodes in myxomatosis of rabbits, *Veterinary Pathology* **11,** 445

Markesich DC, Graham DY, Yoshimura HH (1988) Progress in culture and subculture of spheroplasts and fastidious acid-fast bacilli isolated from intestinal tissues, *Journal of Clinical Microbiology* **26,** 1600-1603

Mason CF, Macdonald SM (1995) Corvids feeding on carrion, *Bird Study* **42,** 255-256

Matthews PRJ, Sargent A (1977) The isolation of mycobacteria from the brown hare (*Lepus europaeus*), *British Veterinary Journal* **133,** 399

McBride JW, Corstvet RE, Taylor BC, Osburn BI (1999) Primary and anamnestic responses of bovine bronchoalveolar and peripheral blood lymphocyte subsets to aerosolized *Pasteurella haemolytica* A1, *Veterinary Immunology and Immunopathology* **67,** 161-170

McClure HM, Chiodini RJ, Anderson DC, Swenson RB, Thayer WR, Coutu JA (1987) *Mycobacterium paratuberculosis* infection in a colony of stumptail macaques (*Macaca arctoides*), *Journal of Infectious Diseases* **155,** 1011-1019

McClure SJ, Davey RL, Emery DL, Colditz IG, Lloyd JB (1996) In vivo depletion of T cells and cytokines during primary exposure of sheep to parasites, *Veterinary Immunology and Immunopathology* **54,** 83-90

McCole DF, Doherty ML, Baird W, Davies WC, McGill K, Torgerson PR (1999) T cell subset involvement in immune responses to *Fasciola hepatica* infection in cattle, *Parasite Immunology* **21,** 1-8

McDonald WL, Ridge SE, Hope AF, Condron RJ (1999) Evaluation of diagnostic tests for Johne's disease in young cattle, *Australian Veterinary Journal* **77,** 113-119

McEwen AD (1939) Investigation on Johne's disease of sheep, *Journal of Comparative Pathology* **52,** 69-87

McFadden JJ, Fidler H (1996) Mycobacteria as possible sources of sarcoidosis and Crohn's disease, *Journal of Applied Bacteriology* **81,** 47S-52S

Mcinnes E, Sopp P, Howard CJ, Taylor G (1999) Phenotypic analysis of local cellular responses in calves infected with bovine respiratory syncytial virus, *Immunology* **96,** 396-403

McNicholl J (1998) Host genes and infectious diseases, *Emerging Infectious Diseases* **4,** 423-426

Medina E, North RJ (1998) Resistance ranking of some common inbred mouse strains to *Mycobacterium tuberculosis* and relationship to major histocompatibility complex haplotype and Nramp1 genotype, *Immunology* **93,** 270-274

Merkal RS, Miller JM, Hintz AM, Bryner JH (1982) Intrauterine inoculation of *Mycobacterium paratuberculosis* into guinea pigs and cattle, *American Journal of Veterinary Research* **43,** 676-678

Merkal RS, Larsen AB, Booth GD (1975) Analysis of the effect of inapparent bovine paratuberculosis, *American Journal of Veterinary Research* **36,** 837-838

Miller DS, Smith BB, Gerros T, Collins MT, Cooley J, McClure J (1999) Paratuberculosis in llamas and alpacas, *Compendium on Continuing Education for the Practicing Veterinarian* **21,** S220-S229

Miller J, Jenny A, Rhyan J, Saari C, Suarez D (1997) Detection of *Mycobacterium bovis* in formalin-fixed, paraffin-embedded tissues of cattle and elk by PCR amplification of an IS6110 sequence specific for *Mycobacterium tuberculosis* complex organisms, *Journal of Veterinary Diagnostic Investigation* **9,** 244-249

Miller MA, Greene CE, Brix AE (1995) Disseminated *Mycobacterium avium intracellulare* complex infection in a miniature schnauzer, *Journal of the American Animal Hospital Association* **31,** 213-216

Milner AR, Mack WN, Coates KJ (1989) A modified ELISA for the detection of goats infected with *Mycobacterium paratuberculosis*, *Australian Veterinary Journal* **66,** 305-307

Miyasaka M, Heron I, Dudler L, Cahill RNP, Forni L, Knaak T, Trnka Z (1983) Studies on the differentiation of T-lymphocytes in sheep. 1. Recognition of a sheep T-lymphocyte differentiation antigen by a monoclonal antibody T-80, *Immunology* **49,** 545-553

Modlin RL, Pirmez C, Hofman FM, Torigian V, Uyemura K, Rea TH, Bloom BR, Brenner MB (1989) Lymphocytes bearing antigen-specific gamma-delta T-cell receptors accumulate in human infectious disease lesions, *Nature* **339,** 544-548

Mokresh AH, Butler DG (1990) Granulomatous enteritis following oral inoculation of newborn rabbits with *Mycobacterium paratuberculosis* of bovine origin, *Canadian Journal of Veterinary Research-Revue Canadienne de Recherche Veterinaire* **54,** 313-319

Mokresh AH, Czuprynski CJ, Butler DG (1989) A rabbit model for study of *Mycobacterium paratuberculosis* infection, *Infection and Immunity* **57,** 3798-3807

Momotani E, Whipple DL, Thiermann AB, Cheville NF (1988) Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ilieal Peyer's patches in calves, *Veterinary Pathology* **25,** 131-137

Mondal D, Sinha RP (1992) Pathogenicity of caprine strain of *Mycobacterium paratuberculosis* in rabbit, *Indian Journal of Animal Sciences* **62,** 1117-1120

Monies RJ, Cranwell MP, Palmer N, Inwald J, Hewinson RG, Rule B (2000) Bovine tuberculosis in domestic cats, *Veterinary Record* **146,** 407-408

Moody DB, Ulrichs T, Muhlecker W, Young DC, Gurcha SS, Grant E, Rosat JP, Brenner MB, Costello CE, Besra GS, Porcelli SA (2000) CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection, *Nature* **404,** 884-888

Moreira AR, Paolicchi F, Morsella C, Zumarraga M, Cataldi A, Fabiana B, Alicia A, Piet O, van Soolingen D, Isabel RM (1999) Distribution of IS900 restriction fragment length polymorphism types among animal *Mycobacterium avium* subsp. *paratuberculosis* isolates from Argentina and Europe, *Veterinary Microbiology* **70,** 251-259

Morishita TY, Fullerton AT, Lowenstine LJ, Gardner IA, Brooks DL (1998) Morbidity and mortality in free-living raptorial birds of northern California: A retrospective study, 1983-1994, *Journal of Avian Medicine and Surgery* **12,** 78-81

Morita CT, Beckman EM, Bukowski JF, Tanaka Y, Band H, Bloom BR, Golan DE, Brenner MB (1995) Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells, *Immunity* **3,** 495-507

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins, *Journal of Immunology* **136,** 2348-2357

Munk ME, Elser C, Kaufmann SHE (1996) Human gamma/delta T-cell response to *Listeria monocytogenes* protein components in vitro, *Immunology* **87,** 230-235

Naser SA, Schwartz D, Shafran I (2000) Isolation of *Mycobacterium avium* subsp *paratuberculosis* from breast milk of Crohn's disease patients, *American Journal of Gasteroenterology* **95,** 1094-1095

Navarro JA, Ramis G, Seva J, Pallares FJ, Sanchez J (1998) Changes in lymphocyte subsets in the intestine and mesenteric lymph nodes in caprine paratuberculosis, *Journal of Comparative Pathology* **118,** 109-121

Neal EG, Cheeseman CL (1991) Badger *Meles meles*, in *A Handbook of British Mammals*, 3rd edition, ed. Corbet GB and Harris S, Blackwell Scientific Press, Oxford p 415-423

Nisbet DI, Gilmour NJL, Brotherston JG (1962) Quantitative studies of *Mycobacterium johnei* in tissues of sheep. III Intestinal histopathology, *Journal of Comparative Pathology* **72,** 80-91

North RJ, LaCourse R, Ryan L, Gros P (1999) Consequence of Nramp1 deletion to *Mycobacterium tuberculosis* infection in mice, *Infection and Immunity* **67,** 5811-5814

O'Neil BD, Pharo HJ (1995) The control of bovine tuberculosis in New Zealand, *New Zealand Veterinary Journal* **43,** 249-255

Ogasawara T, Emoto M, Kiyotani K, Shimokata K, Yoshida T, Nagai Y, Yoshikai Y (1994) Sendai virus pneumonia - evidence for the early recruitment of gamma-delta T-cells during the disease course, *Journal of Virology* **68,** 4022-4027

Oliver JH, Jr. (1996) Lyme borreliosis in the southern United States: a review, *Journal of Parasitology* **82,** 926-935

Orme IM (1993) Immunity to Mycobacteria, *Current Opinion In Immunology* **5,** 497-502

Orme IM, Andersen P, Boom WH (1993) T cell response to *Mycobacterium tuberculosis*, *Journal of Infectious Diseases* **167,** 1481-1497

Ott SL, Wells SJ, Wagner BA (1999) Herd-level economic losses associated with Johne's disease on US dairy operations, *Preventive Veterinary Medicine* **40,** 179-192

Pavlik I, Bejckova L, Pavlas M, Rozsypalova Z, Koskova S (1995) Characterization by restriction-endonuclease analysis and DNA hybridization using IS900 of bovine, ovine, caprine and human dependent strains of *Mycobacterium paratuberculosis* isolated in various localities, *Veterinary Microbiology* **45,** 311-318

Pavlik I, Horvathova A, Dvorska L, Svastova P, DuMaine R, Fixa B, Rychlik I (1999) Homogeneity/heterogeneity of *Mycobacterium avium* subsp *paratuberculosis* strains: correlation between RFLP-type and source (animal, environment, human), in *Proceedings of the Sixth International Colloquium on Paratuberculosis, February 14-18 1999, Melbourne.*, ed. Manning EJB and Collins MT, International Association for Paratuberculosis, Madison, WI p 321-329

Payne JM, Rankin J (1961b) A comparison of the pathogenesis of experimental Johne's disease in calves and cows, *Research in Veterinary Science* **2,** 175-179

Payne JM, Rankin J (1961a) The pathogenesis of experimental Johne's disease in calves, *Research in Veterinary Science* **2,** 167-174

Pearlman E, Kazura JW, Hazlett FE, Jr., Boom WH (1993) Modulation of murine cytokine responses to mycobacterial antigens by helminth-induced T helper 2 cell responses, *Journal of Immunology* **151,** 4857-4864

Perez V, Marin JFG, Badiola JJ (1996) Description and classification of different types of lesion associated with natural paratuberculosis infection in sheep, *Journal of Comparative Pathology* **114,** 107-122

Perez V, Tellechea J, Badiola JJ, Gutierrez M, Marin JFG (1997) Relation between serologic response and pathologic findings in sheep with naturally acquired paratuberculosis, *American Journal of Veterinary Research* **58,** 799-803

Perez V, Tellechea J, Corpa JM, Gutierrez M, Marin JFG (1999) Relation between pathologic findings and cellular immune responses in sheep with naturally acquired paratuberculosis, *American Journal of Veterinary Research* **60,** 123-127

Plante Y, Remenda BW, Chelack BJ, Haines DM (1996) Detection of *Mycobacterium paratuberculosis* in formalin-fixed paraffin-embedded tissues by the polymerase chain reaction, *Canadian Journal of Veterinary Research-Revue Canadienne de Recherche Veterinaire* **60,** 115-120

Pollock JM, Pollock DA, Campbell DG, Girvin RM, Crockard AD, Neill SD, Mackie DP (1996) Dynamic changes in circulating and antigen-responsive T-cell subpopulations post-*Mycobacterium bovis* infection in cattle, *Immunology* **87,** 236-241

Poquet Y, Kroca M, Halary F, Stenmark S, Peyrat MA, Bonneville M, Fournie JJ, Sjostedt A (1998) Expansion of V gamma 9V delta 2 T cells is triggered by *Francisella tularensis*-derived phosphoantigens in tularemia but not after tularemia vaccination, *Infection and Immunity* **66,** 2107-2114

Porcelli S, Brenner MB, Greenstein JL, Balk SP, Terhorst C, Bleicher PA (1989) Recognition of cluster of differentiation-1 antigens by human $CD4^{-}CD8^{-}$ cytolytic T-lymphocytes, *Nature* **341,** 447-450

Porcelli S, Morita CT, Brenner MB (1992) CD1b restricts the response of human CD4-8- T lymphocytes to a microbial antigen, *Nature* **360,** 593-597

Porcelli SA (1995) The CD1 family: a third lineage of antigen-presenting molecules, *Advances in Immunology* **59,** 1-98

Pyrah ITG, Watt NJ (1995) Immunohistological study of the cutaneous delayed-type hypersensitivity reaction in sheep, *Veterinary Immunology and Immunopathology* **48,** 299-312

Quan TJ (1993) Plague and tularemia in rodent populations, in *Zoo and wild animal medicine current therapy 3*, 3rd edition, ed. Fowler ME, W.B. Saunders company, Pennsylvania p 54-56

Qureshi T, Templeton JW, Adams LG (1996) Intracellular survival of *Brucella abortus*, *Mycobacterium bovis* BCG, *Salmonella dublin*, and *Salmonella typhimurium* in macrophages from cattle genetically resistant to *Brucella abortus*, *Veterinary Immunology and Immunopathology* **50,** 55-65

Raadsma HW, Gray GD, Woolaston RR (1998) Breeding for disease resistance in Merino sheep in Australia, *Revue Scientifique et Technique Office International des Epizooties* **17,** 315-328

Radostits OM, Blood DC, Gay CC (1997) Veterinary Medicine. A textbook for the diseases of cattle, sheep, pigs, goats, and horses, 9th edition, ed. Radostits OM, Blood DC, and Gay CC, Bailliere Tindall,

Radzioch D, Kramnik I, Skamene E (1994) Molecular mechanisms of natural-resistance to mycobacterial infections, *Circulatory Shock* **44,** 115-120

Rajasekar R, Sim GK, Augustin A (1990) Self heat-shock and gamma-delta T-cell reactivity, *Proceedings of the National Academy of Sciences of the United States of America* **87,** 1767-1771

Rajya BS, Singh CM (1961) Studies on the pathology of Johne's disease in sheep, *American Journal of Veterinary Research* 189-202

Rankin J (1958) The experimental production of Johne's disease in laboratory rabbits, *Journal of Pathology and Bacteriology* **75,** 363-366

Rankin J (1961) The experimental infection of cattle with *Mycobacterium johnei* III calves maintained in an infectious environment, *Journal of Comparative Pathology* **71,** 10-15

Rankin J (1962) The experimental infection of cattle with *Mycobacterium johnei* IV Adult cattle maintained in an infectious environment, *Journal of Comparative Pathology* **72,** 113-117

Raziuddin S, Telmasani AW, Elawad ME, Alamari O (1992) Gamma-delta T-cells and the immune response in visceral leishmaniasis, *European Journal of Immunology* **22,** 1143-1148

Reddy KP, Sriraman PK, Naidu NRG, Rao PR (1984) Pathology of Johne's disease in sheep, *Indian Veterinary Journal* **61,**

Reid HW (1994) Mycobacterial infections, in *Management and Diseases of Deer; a handbook for the Veterinary Surgeon*, 2rd edition, ed. Alexander TL and Buxton D, Veterinary Deer Society, London p 93-100

Reynaud CA, Mackay CR, Muller RG, Weill JC (1991) Somatic generation of diversity in a mammalian primary lymphoid organ: the sheep ileal Peyer's patches, *Cell* **64,** 995-1005

Reynolds JD, Morris B (1983) The evolution and involution of Peyer's patches in fetal and postnatal sheep, *European Journal of Immunology* **13,** 627-635

Rhind SM, Dutia BM, Howard CJ, Hopkins J (1996) Discrimination of two subsets of CD1 molecules in the sheep, *Veterinary Immunology and Immunopathology* **52,** 265-270

Rhind SM, Hopkins J, Dutia BM (1999) Amino-terminal sequencing of sheep CD1 antigens and identification of a sheep CD1D gene, *Immunogenetics* **49,** 225-230

Richards DT, Harris S, Lewis JW (1995) Epidemiologic studies on intestinal helminth parasites of rural and urban red foxes (*Vulpes vulpes*) in the United Kingdom, *Veterinary Parasitology* **59,** 39-51

Ridge SE, Harkin JT, Badman RT, Mellor AM, Larsen JWA (1995) Johne's disease in alpacas (*Lama pacos*) in Australia, *Australian Veterinary Journal* **72,** 150-153

Ridley DS (1974) Histological classification and the immunological spectrum of leprosy, *Bulletin of the World Health Organisation* **51,** 451-465

Riemann H, Zaman MR, Ruppanner R, Aalund O, Jorgensen JB, Worsaae H, Behymer D (1979) Paratuberculosis in cattle and free-living exotic deer, *Journal of the American Veterinary Medical Association* **174,** 841-843

Ris DR, Hamel KL, Ayling JM (1987) Can sheep become infected by grazing pasture contaminated by cattle with Johne's disease?, *New Zealand Veterinary Journal* **35,** 137

Ris DR, Hamel KL, Weaver AM (1988) Natural transmission of Johne's disease to feral goats, *New Zealand Veterinary Journal* **36,** 98-99

Roger M, Levee G, Chanteau S, Gicquel B, Schurr E (1997) No evidence for linkage between leprosy susceptibility and the human natural resistance-associated macrophage protein 1 (NRAMP1) gene in French Polynesia, *International Journal of Leprosy and other Mycobacterial Diseases* **65,** 197-202

Rossi MC, Gori A, Zehender G, Marchetti G, Ferrario G, De Maddalena C, Catozzi L, Bandera A, Esposti AD, Franzetti F (2000) A PCR-colorimetric microwell plate hybridization assay for detection of *Mycobacterium tuberculosis* and *M. avium* from culture samples and Ziehl-Neelsen-positive smears, *Journal of Clinical Microbiology* **38,** 1772-1776

Roy S, Frodsham A, Saha B, Hazra SK, Mascie-Taylor CG, Hill AV (1999) Association of vitamin D receptor genotype with leprosy type, *Journal of Infectious Diseases* **179,** 187-191

Roy S, McGuire W, MascieTaylor CGN, Saha B, Hazra SK, Hill AVS, Kwiatkowski D (1997) Tumor necrosis factor promoter polymorphism and susceptibility to lepromatous leprosy, *Journal of Infectious Diseases* **176,** 530-532

Rubin JL, Whitlock RH, Habecker PL, Reilly LK, Freeman WA (1999) Hepatic encephalopathy associated with paratuberculosis in a goat, *Journal of the American Veterinary Medical Association* **215,**

Russell DG (1998) What does 'inhibition of phagosome-lysosome fusion' really mean?, *Trends In Microbiology* **6,** 212-214

Sanna E, Woodall CJ, Watt NJ, Clarke CJ, Pittau M, Leoni A, Nieddu AM (2000) In situ-PCR for the detection of *Mycobacterium paratuberculosis* DNA in paraffin-embedded tissues, *European Journal of Histochemistry* **44,** 179-184

Saxegaard F (1981) Serological investigation of *Mycobacterium-avium* and *M avium*-like bacteria isolated from domestic and wild animals, *Acta Veterinaria Scandinavica* **22,** 153-161

Saxegaard F (1985) Om Mykobakterier og mycobakterioser med spesielt henblikk pa nontuberkulose (atypiske) mykobakterier hos dyr i Morge, *Norsk Veterinaertidsskrift* **97,** 547-555

Schaible UE, Collins HL, Kaufmann SHE (1999) Confrontation between Intracellular Bacteria and the Immune System, *Advances in Immunology* **71,** 267-377

Schlienger K, Uyemura K, Jullien D, Sieling PA, Rea TH, Linsley PS, Modlin RL (1998) B7-1 but not CD28, is crucial for the maintenance of the CD4(+) T cell responses in human leprosy, *Journal of Immunology* **161,** 2407-2413

Schneider T, Jahn HU, Liesenfeld O, Steinhoff D, Riecken EO, Zeitz (1997) The number and proportion of V gamma 9V delta 2 T cells rise significantly in the peripheral blood of patients after the onset of acute *Coxiella burnetii* infection, *Clinical Infectious Diseases* **24,** 261-264

Scientific Committee on Animal Health and Animal Welfare (2000) Possible links between Crohn's disease and Paratuberculosis, **16** European Commission, Brussels

Seah GT, Scott GM, Rook GA (2000) Type 2 cytokine gene activation and its relationship to extent of disease in patients with tuberculosis, *Journal of Infectious Diseases* **181,** 385-389

Serraino A, Marchetti G, Sanguinetti V, Rossi MC, Zanoni RC, Catozzi L, Bandera A, Dini W, Mignone W, Franzetti F, Gori A (1999) Monitoring of transmission of tuberculosis between wild boars and cattle: Genotypical analysis of strains by molecular epidemiology techniques, *Journal of Clinical Microbiology* **37,** 2766-2771

Shackelford CC, Reed WM (1989) Disseminated *Mycobacterium avium* infection in a dog, *Journal of Veterinary Diagnostic Investigation* **1,** 273-275

Sharp JM, Stevenson K, Challans JA, Ramage C, Hitchcock D, Reid HW (1997) Mycobacterial infections of free living deer in Scotland, in *Proceedings of the 5th International Colloquium on Paratuberculosis, September 29-October 4, Madison, USA*, ed. Chiodini RJ, Hines ME, and Collins MT, International Association for Paratuberculosis, Madison, WI, USA p 180-182

Sharp JM (2000) Mycobacterial infections, in *Diseases of Sheep*, 3rd edition, ed. Martin WB and Aitken ID, Blackwell Science Ltd, p 143-147

Shibata D (1994) Extraction of DNA from paraffin-embedded tissue for analysis by polymerase chain-reaction - new tricks from an old friend, *Human Pathology* **25,** 561-563

Shields ED, Russell DA, Pericakvance MA (1987) Genetic epidemiology of the susceptibility to leprosy, *Journal of Clinical Investigation* **79,** 1139-1143

Shinkai K, Locksley RM (2000) CD1, tuberculosis, and the evolution of major histocompatibility complex molecules, *Journal of Experimental Medicine* **191,** 907-914

Shulaw WP, Bechnielsen S, Rings DM, Getzy DM, Woodruff TS (1993) Serodiagnosis of paratuberculosis in sheep by use of agar gel immunodiffusion, *American Journal of Veterinary Research* **54,** 13-19

Sieling PA, Chatterjee D, Porcelli SA, Prigozy TI, Mazzaccaro RJ, Soriano T, Bloom BR, Brenner MB, Kronenberg M, Brennan PJ, Modlin RL (1995) CD1-restricted T-cell recognition of microbial lipoglycan antigens, *Science* **269,** 227-230

Sieling PA, Jullien D, Dahlem M, Tedder TF, Rea TH, Modlin RL, Porcelli SA (1999) CD1 expression by dendritic cells in human leprosy lesions: Correlation with effective host immunity, *Journal of Immunology* **162,** 1851-1858

Sieling PA, Ochoa MT, Jullien D, Leslie DS, Sabet S, Rosat JP, Burdick AE, Rea TH, Brenner MB, Porcelli SA, Modlin RL (2000) Evidence for human CD4+ T cells in the CD1-restricted repertoire: derivation of mycobacteria-reactive T cells from leprosy lesions, *Journal of Immunology* **164,** 4790-4796

Sigurdardottir OG, Press CM, Saxegaard F, Evensen O (1999) Bacterial isolation, immunological response, and histopathological lesions during the early subclinical phase of experimental infection of goat kids with *Mycobacterium avium* subsp *paratuberculosis*, *Veterinary Pathology* **36,** 542-550

Sigurdsson B (1960) A killed vaccine against paratuberculosis (Johne's disease) in sheep, *American Journal of Veterinary Research* **21,** 54-67

Smit T, Eger A, Haagsma J, Bakhuizen T (1987) Avian tuberculosis in wild birds in the Netherlands, *Journal of Wildlife Diseases* **23,** 485-487

Smith RA, Kreeger JM, Alvarez AJ, Goin JC, Davis WC, Whipple DL, Estes DM (1999) Role of CD8(+) and WC-1(+) gamma/delta T cells in resistance to *Mycobacterium bovis* infection in the SCID-bo mouse, *Journal of Leukocyte Biology* **65,** 28-34

Soltys J, Quinn MT (1999) Selective recruitment of T-cell subsets to the udder during staphylococcal and streptococcal mastitis: Analysis of lymphocyte subsets and adhesion molecule expression, *Infection and Immunity* **67,** 6293-6302

Spada FM, Grant EP, Peters PJ, Sugita M, Melian A, Leslie DS, Lee HK, van Donselaar E, Hanson DA, Krensky AM, Majdic O, Porcelli SA, Morita CT, Brenner MB (2000) Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity, *Journal of Experimental Medicine* **191,** 937-948

Spangler E, Heider LE, Bech-Nielsen S, Dorn CR (1991) Serologic enzyme-linked immunosorbent assay responses of calves vaccinated with a killed *Mycobacterium paratuberculosis* vaccine, *American Journal of Veterinary Research* **52,** 1197-1200

Stabel JR (1996) Production of gamma-interferon by peripheral blood mononuclear cells: An important diagnostic tool for detection of subclinical paratuberculosis, *Journal of Veterinary Diagnostic Investigation* **8,** 345-350

Stabel JR (2000) Cytokine secretion by peripheral blood mononuclear cells from cows infected with *Mycobacterium paratuberculosis*, *American Journal of Veterinary Research* **61,** 754-760

Stabel JR, Ackermann MR, Goff JP (1996b) Comparison of polyclonal antibodies to three different preparations of *Mycobacterium paratuberculosis* in immunohistochemical diagnosis of Johne's disease in cattle, *Journal of Veterinary Diagnostic Investigation* **8,** 469-473

Stabel JR, Goff JP, Whipple DL, Ackermann MR, Reinhardt TA (1996a) Low calcium diet and 1,25-dihydroxyvitamin D(3) infusion modulate immune responses during Mycobacterium paratuberculosis infection in beige mice, *Veterinary Immunology and Immunopathology* **50,** 127-143

Stamp JT, Watt JA (1954) Johne's disease in sheep, *Journal of Comparative Pathology* **64,** 26-39

Stenger S, Niazi KR, Modlin RL (1998) Down-regulation of CD1 on antigen-presenting cells by infection with *Mycobacterium tuberculosis*, *Journal of Immunology* **161,** 3582-3588

Stevenson K, Sharp JM (1997) The contribution of molecular biology to *Mycobacterium avium* subspecies *paratuberculosis* research, *Veterinary Journal* **153,** 269-286

Stuart P (1965) Vaccination against Johne's disease in cattle exposed to experimental infection, *British Veterinary Journal* **121,** 289-318

Sugita M, Moody DB, Jackman RM, Grant EP, Rosat JP, Behar SM, Peters PJ, Porcelli SA, Brenner MB (1998) CD1 - A new paradigm for antigen presentation and T cell activation, *Clinical Immunology and Immunopathology* **87,** 8-14

Sugita M, Peters P, Brenner MB (2000b) Pathways for lipid antigen presentation by CD1 molecules: nowhere for intracellular pathogens to hide, *Traffic* **1,** 295-300

Sugita Y, Ishii N, Katsuno M, Yamada R, Nakajima H (2000a) Familial cluster of cutaneous *Mycobacterium avium* infection resulting from use of a circulating, constantly heated bath water system, *British Journal of Dermatology* **142,** 789-793

Sung N, Collins MT (1998) Thermal tolerance of *Mycobacterium paratuberculosis*, *Applied And Environmental Microbiology* **64,** 999-1005

Sweeney RW, Jones DE, Habecker P, Scott P (1998) Interferon-gamma and interleukin 4 gene expression in cows infected with *Mycobacterium paratuberculosis*, *American Journal of Veterinary Research* **59,** 842-847

Szalay G, Zugel U, Ladel CH, Kaufmann SHE (1999) Participation of group 2 CD1 molecules in the control of murine tuberculosis, *Microbes and Infection* **1,** 1153-1157

Tanaka S, Itohara S, Sato M, Taniguchi T, Yokomizo Y (2000) Reduced formation of granulomata in gamma(delta) T cell knockout BALB/c mice inoculated with *Mycobacterium avium* subsp. *paratuberculosis*, *Veterinary Pathology* **37,** 415-421

Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR (1995) Natural and synthetic non-peptide antigens recognized by human gamma delta T cells, *Nature* **375,** 155-158

Taylor AW (1953a) Experimental Johne's disease in cattle, *Journal of Comparative Pathology* **63,** 355-367

Taylor AW (1953b) The experimental infection of cattle with varieties of *Mycobacterium johnei* isolated from sheep, *Journal of Comparative Pathology* **63,** 368-373

Teitelbaum R, Schubert W, Gunther L, Kress Y, Macaluso F, Pollard JW, McMurray DN, Bloom BR (1999) The M cell as a portal of entry to the lung for the bacterial pathogen *Mycobacterium tuberculosis*, *Immunity* **10,** 641-650

Thoen CO, Haagsma J (1996) Molecular techniques in the diagnosis and control of paratuberculosis in cattle, *Journal of the American Veterinary Medical Association* **209,** 734-737

Thoen CO, Himes EM, Barrett RE (1977) *Mycobacterium avium* serotype 1 infection in a sandhill crane (*Grus canadensis*), *Journal of Wildlife Diseases* **13,** 40-42

Thompson DE (1994) The role of Mycobacteria in Crohns-disease, *Journal of Medical Microbiology* **41,** 74-94

Thompson K, Gwozdz J, West D, Murray A (1999) Johne's disease, *Wool Technology and Sheep Breeding* **47,** 66-70

Thorel MF, Krichevsky M, Levy-Frebault VV (1990) Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. *nov.*, *Mycobacterium avium* subsp. *paratuberculosis* subsp. *nov.*, and *Mycobacterium avium* subsp. *silvaticum* subsp. *nov*, *International Journal of Systematic Bacteriology* **40,** 254-260

Todd JR, West BC, Mcdonald JC (1990) Human-leukocyte antigen and leprosy - study in Northern Louisiana and review, *Reviews of Infectious Diseases* **12,** 63-74

Torres M, Herrera T, Villareal H, Rich EA, Sada E (1998) Cytokine profiles for peripheral blood lymphocytes from patients with active pulmonary tuberculosis and healthy household contacts in response to the 30-kilodalton antigen of *Mycobacterium tuberculosis*, *Infection and Immunity* **66,** 176-180

Tsukaguchi K, Balaji KN, Boom WH (1995) CD4+ alpha beta T cell and gamma delta T cell responses to *Mycobacterium tuberculosis*. Similarities and differences in Ag recognition, cytotoxic effector function, and cytokine production, *Journal of Immunology* **154,** 1786-1796

Twort C (1914) The experimental production of Johne's disease in some of the laboratory animals, *The Veterinary News* 78-79

Vago L, Barberis M, Gori A, Scarpellini P, Sala E, Nebuloni M, Bonetto S, Cannone M, Marchetti G, Franzetti F, Costanzi G (1998) Nested polymerase chain reaction for *Mycobacterium tuberculosis* IS6110 sequence on formalin-fixed paraffin-embedded tissues with granulomatous diseases for rapid diagnosis of tuberculosis, *American Journal of Clinical Pathology* **109,** 411-415

van Soolingen D, van der Zanden AG, de Haas PE, Noordhoek GT, Kiers A, Foudraine NA, Portaels F, Kolk AH, Kremer K, van Embden JD (1998) Diagnosis of *Mycobacterium microti* infections among humans by using novel genetic markers, *Journal of Clinical Microbiology* **36,** 1840-1845

vanCrevel R, Karyadi E, Preyers F, Leenders M, Kullberg BJ, Nelwan RHH, vanderMeer JWM (2000) Increased production of interleukin 4 by CD4(+) and CD8(+) T cells from patients with tuberculosis is related to the presence of pulmonary cavities, *Journal of Infectious Diseases* **181,** 1194-1197

Vankruiningen HJ (1999) Lack of support for a common etiology in Johne's disease of animals and Crohn's disease in humans, *Inflammatory Bowel Diseases* **5,** 183-191

Veazey RS, Horohov DW, Krahenbuhl JL (1995b) Comparison of the resistance of c57bl/6 and c3h/he mice, *Veterinary Microbiology* **47,** 79-87

Veazey RS, Taylor HW, Horohov DW, Krahenbuhl JL, Oliver JL, III, Snider TG, III (1995a) Histopathology of C57BL/6 mice inoculated orally with *Mycobacterium paratuberculosis*, *Journal of Comparative Pathology* **113,** 75-80

Vidal S, Tremblay ML, Govoni G, Gauthier S, Sebastiani G, Malo D, Skamene E, Olivier M, Jothy S, Gros P (1995) The Ity/Lsh/Bcg locus - natural resistance to infection with intracellular parasites is abrogated by disruption of the NRAMP1 gene, *Journal of Experimental Medicine* **182,** 655-666

Vidal SM, Malo D, Vogan K, Skamene E, Gros P (1993) Natural resistance to infection with intracellular parasites - isolation of a candidate for Bcg, *Cell* **73,** 469-485

Waite RK (1985) Food caching and recovery by farmland corvids, *Bird Study* **32,** 45-49

Wall S, Kunze ZM, Saboor S, Soufleri I, Seechurn P, Chiodini R, McFadden JJ (1993) Identification of spheroplast-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction, *Journal of Clinical Microbiology* **31,** 1241-1245

Waters WR, Stabel JR, Sacco RE, Harp JA, Pesch BA, Wannemuehler MJ (1999) Antigen-specific B-cell unresponsiveness induced by chronic *Mycobacterium avium* subsp. *paratuberculosis* infection of cattle, *Infection and Immunity* **67,** 1593-1598

Webster JP, Macdonald DW (1995) Parasites of wild brown rats (*Rattus norvegicus*) on UK farms, *Parasitology* **111 (Pt 3),** 247-255

Wells AQ (1937) Tuberculosis in wild voles, *Lancet* 1221

Wells SJ, Wagner BA (2000) Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures, *Journal of American Veterinary Medical Association* **216,** 1450-1457

Whipple DL, Callihan DR, Jarnagin JL (1991) Cultivation of *Mycobacterium paratuberculosis* from bovine fecal specimens and a suggested standardized procedure, *Journal of Veterinary Diagnostic Investigation* **3,** 368-373

Whist SK, Storset AK, Larsen HJS (2000) The use of interleukin-2 receptor expression as a marker of cell-mediated immunity in goats experimentally infected with *Mycobacterium avium* ssp *paratuberculosis*, *Veterinary Immunology and Immunopathology* **73,** 207-218

Whitlock RH, Rosenberger AE, Sweeney RW, Spencer PA (1996) Distribution of *M. paratuberculosis* in tissues of cattle from herds and infected with Johne's disease, in *Proceedings of the 5th International Colloquium on Paratuberculosis, September 29-October 4, Madison, USA*, ed. Chiodini RJ, Hines ME, and Collins MT, International Association for Paratuberculosis, Madison, WI p 168-174

Whittington R, Marsh I, Choy E, Cousins D (1998a) Polymorphisms in IS1311, an insertion sequence common to *Mycobacterium avium* and *M. avium* subsp. *paratuberculosis*, can be used to distinguish between and within these species, *Molecular And Cellular Probes* **12,** 349-358

Whittington RJ (2000) Molecular epidemiological confirmation of the occurrence of sheep (S) strains of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in Australia and sheep and cattle in Iceland, *Veterinary Microbiology* **in press,**

Whittington RJ, Fell S, Walker D, McAllister S, Marsh I, Sergeant E, Taragel CA, Marshall DJ, Links IJ (2000a) Use of Pooled Fecal Culture for Sensitive and Economic Detection of *Mycobacterium avium* subsp. *paratuberculosis* Infection in Flocks of Sheep, *Journal of Clinical Microbiology* **38,** 2550-2556

Whittington RJ, Hope AF, Marshall DJ, Taragel CA, Marsh I (2000b) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: IS900 restriction fragment length polymorphism and IS1311 polymorphism analyses of isolates from animals and a human in Australia, *Journal of Clinical Microbiology* **38,** 3240-3248

Whittington RJ, Marsh I, McAllister S, Turner MJ, Marshall DJ, Fraser CA (1999b) Evaluation of modified BACTEC 12B radiometric medium and solid media for culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep, *Journal of Clinical Microbiology* **37,** 1077-1083

Whittington RJ, Marsh I, Turner MJ, McAllister S, Choy E, Eamens GJ, Marshall DJ, Ottaway S (1998b) Rapid detection of *Mycobacterium paratuberculosis* in clinical samples from ruminants and in spiked environmental samples by modified BACTEC 12B radiometric culture and direct confirmation by IS900 PCR, *Journal of Clinical Microbiology* **36,** 701-707

Whittington RJ, Reddacliff L, Marsh I, Saunders V (1999a) Detection of *Mycobacterium avium* subsp *paratuberculosis* in formalin- fixed paraffin-embedded intestinal tissue by IS900 polymerase chain reaction, *Australian Veterinary Journal* **77,** 392-397

Wijngaard PLJ, Metzelaar MJ, Machugh ND, Morrison WI, Clevers HC (1992) Molecular characterisation of the WC1 antigen expressed specifically on bovine CD4-CD8- gamma delta T lymphocytes, *Journal of Immunology* **149,** 3273-3277

Wilesmith JW (1991) Epidemiological methods for investigating wild animal reservoirs of animal disease, *Revue Scientifique et Technique Office International des Epizooties* **10,** 205-214

Wilesmith JW, Sayers PE, Little TWA, Brewer JI, Bode R, Hillman GDB, Pritchard DG, Stuart FA (1986) Tuberculosis in East Sussex IV. A systematic examination of wild mammals other than badgers for tuberculosis, *Journal of Hygiene (Cambridge)* **97,** 37-48

Williams ES, Spraker TR, Schoonveld GG (1979) Paratuberculosis (Johne's disease) in bighorn sheep and a rocky mountain goat in Colorado, *Journal of Wildlife Diseases* **15,** 221-227

Withers FW (1959) 2. Incidence of the disease, *Veterinary Record* **71,** 1150-1153

Wright SD (1995) CD14 and innate recognition of bacteria, *Journal of Immunology* **155,** 6-8

Yamada G, Shijubo N, Shigehara K, Okamura H, Kurimoto M, Abe S (2000) Increased levels of circulating interleukin-18 in patients with advanced tuberculosis, *American Journal of Respiratory and Critical Care Medicine* **161,** 1786-1789

Yamamura M, Uyemura K, Deans RJ, Weinberg K, Rea TH, Bloom BR, Modlin RL (1991) Defining protective responses to pathogens - cytokine profiles in leprosy lesions, *Science* **254,** 277-279

Zeng Z, Castano AR, Segelke BW, Stura EA, Peterson PA, Wilson IA (1997) Crystal structure of mouse CD1: An MHC-like fold with a large hydrophobic binding groove, *Science* **277,** 339-345

Zurbrick BG, Czuprynski CJ (1987) Ingestion and intracellular growth of *Mycobacterium paratuberculosis* within bovine blood monocytes and monocyte dervived macrophages, *Infection and Immunity* **55,** 1588-1593

Zwilling BS, Kuhn DE, Wikoff L, Brown D, Lafuse W (1999) Role of iron in Nramp1-mediated inhibition of mycobacterial growth, *Infection and Immunity* **67,** 1386-1392

Appendix One: FACS results from experiment 6.3

Results are expressed as the number of cells (%) in each tissue, as an average for each group. Statistically significant results ($p<0.05$) are marked with an asterisk.

Data from PBL

Pre experiment (start of week 1)

	B cells	CD4	CD8	γδ TCR
control	17.37	18.97	20.65	22.94
SEM	3.12	5.3	3.58	1.42
heat-killed	21.44	13.39	24.01	25.41
SEM	2.66	2.14	2.54	2.79
live infected	24.88	14.42	26.96	22.27
SEM	4.75	2.03	2.68	1.98

Week 2

	B cells	CD4	CD8	γδ TCR
control	17.66	9.77	26.88	19.74
SEM	4.55	0.817	3.25	0.815
heat-killed	16.74	11.81	25.14	18.06
SEM	2.74	0.903	1.59	2.71
live infected	24.99	12.83	25.67	17.09
SEM	5.18	1.86	3.6	1.65

Week 3

	B cells	CD4	CD8	γδ TCR
control	26.04	12.89	29.47	25.35
SEM	3.69	0.462	4.56	3.32
heat-killed	25.42	19.84	26	29.83
SEM	4.8	4.51	2.76	5.47
live infected	31.65	14.4	27.96	19.84
SEM	5.32	1.52	3.08	2.08

Week 4

	B cells	CD4	CD8	γδ TCR
control	26.7	12.31	27.92	22.25
SEM	2.47	1.7	3.32	2.43
heat-killed	29.52	9.889	20.78	22.99
SEM	3.46	0.803	1.01	2.91
live infected	28.14	11.5	25.91	18.15
SEM	3.69	1.23	1.95	2.64

Data from post mortem tissues

PBL taken at time of post mortem

	CD4	CD8	B cells	γδ TCR
control	9.27	20.57	36.78	22.36
SEM	1.12	2.4	6.26	1.75
heat-killed	9.67	13.31*[1]	35.4	14.88*[2]
SEM	1.54	2.95	6.06	1.6
live infected	13.24	19.26	49.02	17.89
SEM	3.03	3.89	5.53	3.4

[1]p=0.035 [2]p=0.005

JPP

	CD4	CD8	B cells	γδ TCR
control	23.71	43.39	32.47	4.26
SEM	1.14	4.86	3.45	2.17
heat-killed	23.97	33.81	29.98	2.21
SEM	1.61	4.16	4.65	0.776
live infected	23.82	39.61	29.19	4.03
SEM	1.4	3.07	3.36	1.28

IPP

	CD4	CD8	B cells	γδ TCR
control	1.67	2.86	76.59	0.35
SEM	0.56	0.82	3.21	0.239
heat-killed	2.42	2.44	75.49	1.8
SEM	1.66	0.814	3.24	1.07
live infected	1.5	3.111	75.59	0.778
SEM	0.483	0.561	2.99	0.565

MLN

	CD4	CD8	B cells	γδ TCR
control	32.68	61.47	21.51	7.57
SEM	1.79	3.08	1.75	1.4
heat-killed	33.85	66.91	19.08	10.6
SEM	1.34	1.96	1.75	2.03
live infected	34.73	64.64	21.08	7.7
SEM	2.87	2.83	2.16	1.15

PLN

	CD4	CD8	B cells	γδ TCR
control	35.78	61.9	16.98	8.84
SEM	2.41	3.52	1.97	1.18
heat-killed	38.2	69.85	13.16	8.29
SEM	2.47	2.69	1.42	0.904
live infected	42.3	59.45	14.27	8.47
SEM	2.99	5.46	2.06	1.13

Appendix Two: Suppliers Details

ABgene
Blenheim Road
Epsom
Surrey KT19 9AP
www.adbio.co.uk

Allied Monitor
P.O. Box 71
201 Golden Drive
Fayette
Missouri 65248
USA

Amersham International plc
Amersham Place
Little Chalfont
Bucks HP7 9BR
UK
www.apbiotech.com

Bayer plc
Animal Health Business Group
Eastern Way
Bury St Edmunds
Suffolk IP32 7AH
UK
www.bayerus.com

BDH
Merck House
Poole
Dorset BH15 1TD
UK
www.merckeurolab.ltd.uk

Becton-Dickinson
Between Towns Road
Cowley
Oxford OX4 3LY
UK
www.bd.com

bio-Merieux, Inc.
St Louis
Missouri
USA
www.biomerieux-vitek.com

Bio-Rad Laboratories Ltd.
Bio-Rad House
Maylands Avenue
Hemel Hempstead
Herts HP2 7TD
UK
www.bio-rad.com

BioSpec Products
P.O. Box 722
Bartlesville
Oklahoma 74005
USA

Boehringer Mannheim
Roche Diagnostics Ltd.
Bell Lane
Lewes
East Sussex BN7 1LG
www.biochem.boehringer-mannheim.com

DAKO Ltd
Denmark House
Angel Drove
Ely
Cambridgeshire CB7 4ET
www.dakoltd.co.uk

Difco Laboratories
P.O. Box 14B
Central Avenue
West Molesey
Surrey KT8 2SE
UK

Dynex Technologies
Action Court
Ashford Road
Ashford
Middlesex TN15 1XB
www.dynextechnologies.com

Fort Dodge Animal Health
Flanders Road
Hedge End
Southampton SO30 4QH
UK
www.ahp.com/fortdodge.htm

Hybaid Ltd.
St Clare Business Park
Holly Road
Hampton Hill
Middlesex TW12 IP2
UK
www.hybaid.co.uk

Life Technologies Ltd.
2 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF
UK
www.lifetech.com

Martindale Pharmaceuticals Ltd,
Bampton Rd
Romford RM3 8UG
UK

Mast Laboratories Ltd.
Mast House
Derby Road
Bootle
Merseyside L20 1EA
UK

Nycomed Ltd.
2111 Coventry Road
Sheldon
Birmingham B26 3EA
UK

Oswel Ltd.
Medical and Biological Sciences Building
University of Southampton
Boldrewood
Bassett Crescent East
Southampton SO16 7PX

Pfizer Limited
Ramsgate Road
Sandwich
Kent CT13 9NJ
UK
www.pfizer.com

Promega UK

Delta House
Chilworth Research Centre
Southampton SO16 7NS
UK
www.promega.com

Qiagen Ltd.
Unit 1
Tillingbourne Court
Dorking Business Centre
Dorking
Surrey RH4 1HJ
UK
www.qiagen.com

Schering-Plough Animal Health
Division of Schering-Plough Ltd
Breakspear Road South
Harefield
Uxbridge
Middlesex UB9 6LS
UK
www.schering-plough.com

Scotlabs
Kirkshaws Road
Coatbridge
Lanarkshire ML5 5AD
UK

Scottish Antibody Production Unit
Lanarkshire
UK

Seward Medical
Great North Road
London N2 0GN
UK
www.seward.co.uk

Sigma-Aldrich Co. Ltd.
Fancy Road
Poole
Dorset BH12 4QH
UK
www.sigma-aldrich.com

Vector Labs
3 Accent Park

Bakewell Road
Orton SOuthgate
Peterborough PE2 6XS
UK
www.vectorlabs.com

Virbac Limited
Woolpit Business Park
Windmill Avenue
Woolpit
Bury St Edmunds
Suffolk IP30 9UP
UK
www.virbac.co.uk

Appendix Three: Publications

Refereed publications:

Beard PM, Henderson D, Daniels MJ, Pirie A, Buxton D, Greig A, Hutchings MR, McKendrick I, Rhind S, Stevenson K, Sharp JM (1999) Evidence of paratuberculosis in fox (*Vulpes vulpes*) and stoat (*Mustela erminea*), *Veterinary Record* **145,** 612-613

Beard PM, Rhind S, Sinclair MC, Wildblood LA, Stevenson K, McKendrick IJ, Sharp JM, Jones DG, (2000) Modulation of γδ T cells and CD1 in *Mycobacterium avium* subsp *paratuberculosis* infection, *Veterinary Immunology and Immunopathology* **77**, 311-319

Beard PM, Daniels MJ, Henderson D, Pirie A, Rudge K, Buxton D, Rhind S, Greig A, Hutchings MR, McKendrick I, Stevenson K, Sharp JM Paratuberculosis Infection of Non-ruminant Wildlife in Scotland *Journal of Clinical Microbiology* in press

Beard PM, Rhind S, Buxton D, Daniels MJ, Henderson D, Pirie A, Rudge K, Greig A, Hutchings MR, Stevenson K, Sharp JM Natural Paratuberculosis Infection in Rabbits in Scotland *Journal of Comparative Pathology* in press

Non-refereed publications:

Beard PM, Rhind S, Buxton D, Daniels MJ, Henderson D, Pirie A, Rudge K, Greig A, Hutchings MR, Stevenson K, Sharp JM (2000) Wildlife reservoirs of paratuberculosis *Research into Veterinary Science*, supp. A, page 2, BA04

Beard PM (2000) Paratuberculosis in wildlife *Investigation - the newsletter of the British Wildlife Association* **3**, 9.

Sinclair MC, Wildblood LA, Stevenson K, Beard P, Sharp JM, Hopkins J & Jones DG (1999) The initial T-cell and cytokine profile of lambs experimentally infected with mycobacteria. *Immunology* **98**, 16.5

Beard PM (1999) The role of non-farmed animals and Johne's disease. In *Report on the Sixth International Colloquium on Paratuberculosis - Researcher summary of key issues and implications for Johne's disease in Australia* p31-37.

Beard P, Hopkins J, Rhind S, Stevenson K and Sharp JM (1999). Role of Nramp 1 in Johne's disease. In *Proceedings of the Sixth International Colloquium on Paratuberculosis.* (Manning, E.J.B. and Collins, M.T. eds.) Madison, WI: International Association for Paratuberculosis Inc. p675.

Appendix Four: Abbreviations used

AFB – acid fast bacteria
AGID – agar gel imunodiffusion
APC – antigen presenting cell
APM – antigen presenting molecule
BCG – bacillus Calmette-Guerin
BVDV – bovine viral diarrhoea virus
CAEV – caprine arthritis and encephalitis virus
cfu – colony forming units
cmi – cell mediated immunity
DNA – deoxyribonucleic acid
DTH – delayed type hypersensitivity
ELISA – enzyme-linked immunosorbent assay
FAE – follicle associated epithelium
ffpe – formalin fixed paraffin embedded
GALT – gut associated lymphoid tissue
GM-CSF – granulocyte macrophage colony stimulating factor
H&E – haematoxylin and eosin
ICLN – ileocaecal lymph node
ICV – ileocaecal valve
IFN-γ - gamma interferon
IL – interleukin
IPP – ileal Peyer's patch
JPP – jejunal Peyer's patch
KO – knock out
M.a. avium – *Mycobacterium avium* subsp *avium*
M.a. paratuberculosis - *Mycobacterium avium* subsp *paratuberculosis*
M.a. sylvaticum – *Mycobacterium avium* subsp *sylvaticum*
MHC – major histocompatibility complex
MLN – mesenteric lymph node
mRNA – messenger ribonucleic acid
PBL – peripheral blood lymphocytes
PCR – polymerase chain reaction
pi – post infection
PFGE – pulsed field gel electrophoresis
RFLP – restriction fragment length polymorphism
RNA – ribonucleic acid
TCR – T cell receptor
TGF-β - transforming growth factor beta
TNF-α - tumour necrosis factor alpha
UK – United Kingdom
ZN - Ziehl Neelsen

Evidence of paratuberculosis in fox (*Vulpes vulpes*) and stoat (*Mustela erminea*)

P. M. BEARD, D. HENDERSON, M. J. DANIELS, A. PIRIE, D. BUXTON, A. GREIG, M. R. HUTCHINGS, I. MCKENDRICK, S. RHIND, K. STEVENSON, J. M. SHARP

JOHNE'S disease, or paratuberculosis, is a chronic enteritis of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (*M a paratuberculosis*). Clinical signs of Johne's disease include weight loss, decreased production, and eventual death. There is no effective treatment and no cure. Until recently, *M a paratuberculosis* was thought to cause natural infection in ruminants only. Johne's disease is found worldwide in domestic ruminants, and has also been reported in wild and captive ruminants, including saiga antelope (*Saiga tatarica*) (Dukes and others 1992), bighorn sheep (*Ovis canadensis*) and Rocky Mountain goats (*Oreamnos americanus*) (Williams and others 1983), and wild deer (Riemann and others 1979, Sharp and others 1996). Although Johne's disease has been present in the UK sheep and cattle population for many years, there is still relatively little known about the epidemiology of this important disease. There is mounting evidence for a much wider host range than had been prevously recognised, including non-ruminants. Natural infection has now been documented in macaque monkeys (McClure and others 1987) and rabbits. Recent studies in Scotland (Greig and others 1997) have found *M a paratuberculosis* infecting the intestinal tract of rabbits. A strong statistical association was found between Johne's disease in rabbits and a history of Johne's disease in cattle on affected farms (Greig and others 1999). This evidence suggests that rabbits may play a role as wildlife reservoirs of the disease. This short communication reports preliminary findings from a wider study investigating other wildlife, especially carnivorous species, to delineate further the host range of Johne's disease.

Nine foxes (*Vulpes vulpes*) from four farms (A, B, C and D), and five stoats (*Mustela erminea*) from a separate farm (E) were collected as part of the farm vermin control programme. Farms A, B and E had Johne's disease in both the ruminant livestock and rabbit populations; farms C and D were in the close vicinity of an affected farm. Farms A, B and D were in the Perth and Kinross region, and farms C and E in Angus. Postmortem examination was carried out, and samples of liver, jejunum, terminal ileum, ileocaecal junction, caecum, colon and mesenteric lymph node were collected for culture and histopathology. The samples for histopathology were fixed in 10 per cent buffered formol saline for a minimum of 48 hours, trimmed, dehydrated through graded alcohols, embedded in paraffin wax and 5 µm thick sections were cut. These were stained with haematoxylin and eosin for routine histopathological examination, and for acid-fast bacilli (AFB) by the Ziehl-Neelsen method. Samples for culture were pooled for each animal and processed as described by Greig and others (1999). The identity of mycobacterial isolates was confirmed by polymerase chain reaction (Greig and others 1999). This technique allowed detection of the IS900 insertion sequence which is found only in *M a paratuberculosis*.

M a paratuberculosis was cultured from the tissues of eight foxes (89 per cent) and three stoats (60 per cent) from the five farms. The histopathological lesions showed single macrophage-like cells or discrete granulomata consisting of small numbers of cells with the appearance of macrophages, in the cortex and paracortex of the mesenteric lymph node of six of the nine foxes. Small numbers of intracellular AFB were present in the individual macrophages, and irregularly scattered in the granulomata (Fig 1). Similar lesions were present in the mesenteric lymph node of one stoat (Table 1).

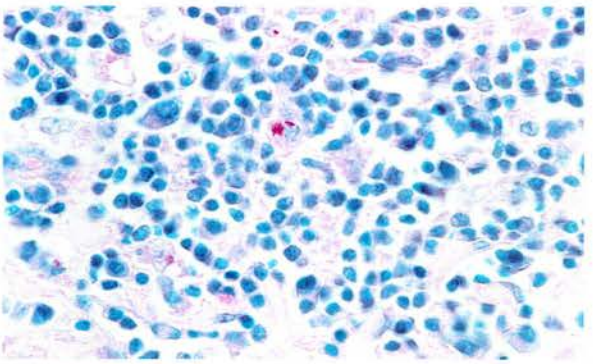


FIG 1: Ziehl-Neelsen stained section of mesenteric lymph node from a fox, showing intracellular acid-fast rods. × 1000

TABLE 1: Summary of culture and histopathological results

Farm	Animals examined	Number positive on culture	Presence of AFB	Presence of Histological lesions
A	4 foxes	3	3	3
B	1 fox	1	0	0
C	1 fox	1	1	1
D	3 foxes	3	2	2
E	5 stoats	3	1	1

AFB Acid-fast bacillli

These findings are similar to descriptions of early ovine and bovine Johne's disease lesions, which comprise an occasional giant cell or epithelioid cell in the villous lamina propria or paracortical areas of lymph nodes, with very few AFB present (Buergelt and others 1978, Perez and others 1996). The discrepancies between culture and histopathological results in this study are to be expected, due to the mild pathological changes, and the recognised difficulties in culturing *M a paratuberculosis*. No lesions were observed in samples of intestine or liver, which showed varying degrees of autolysis, preventing an assessment of subtle pathological changes.

Numerous studies have shown that rabbits are frequently eaten by both foxes and stoats (Harris and Lloyd 1991, King 1991); consequently, passive carriage of *M a paratuberculosis* by the foxes and stoats remains a possible explanation for the positive culture results. However, the detection of AFB and chronic inflammatory cells in lymphoid tissue provides strong evidence for natural infection.

This is the first reported isolation of *M a paratuberculosis* from either fox or stoat. Until recently, natural *M a paratuberculosis* infection was considered only to occur in ruminants. However, these results support the suggestion that *M a paratuberculosis* has a wider host range, and that Johne's disease may have a more complex epidemiology, than has been assumed previously. Rabbit, fox, stoat and possibly other species may harbour *M a paratuberculosis*, and act as a reservoir of infection, perpetuating the cycle of disease on the farm. Thorough understanding of all potential wildlife reservoirs is necessary for the development of informed control programmes to combat Johne's disease. Further studies are in progress to investigate the role of wildlife in the epidemiology of Johne's disease.

ACKNOWLEDGEMENTS

The authors would like to thank Val Forbes and Alison Baird for the histopathological sections, the landowners involved in

Veterinary Record (1999) **145,** 612-613

P. M. Beard, BVSc, MRCVS, **A. Pirie,** BSc, **D. Buxton,** BVM&S, PhD, FRCVS, FRCPath, **K. Stevenson,** BSc, PhD, **J. M. Sharp,** BVMS, PhD, MRCVS, Moredun Research Institute, International Research Centre, Pentlands Science Park, Bush Loan, Penicuik, Midlothian EH26 0PZ
D. Henderson, FIBMS, **A. Greig,** BVM&S, FRCVS, Veterinary Science Division, Scottish Agricultural College, Cleeve Gardens, Oakbank Road, Perth PH1 1HF
I. McKendrick, PhD, Biomathematics and Statistics Scotland, The King's Buildings, Edinburgh EH9 3JZ
S. Rhind, BVMS, PhD, MRCVS, Department of Veterinary Pathology, Easter Bush Veterinary Centre, University of Edinburgh, Bush Loan, Midlothian EH26 0PZ
M. J. Daniels, BSc, PhD, **M. R. Hutchings,** BSc, MSc, PhD, Animal Biology Division, Scottish Agricultural College, West Mains Road, Edinburgh EH9 3JG

the study, and Brian Easter for photographic assistance. This project was funded by the Scottish Executive Rural Affairs Department.

References

BUERGELT, C. D., HALL, C., McENTREE, K. & DUNCAN, J. R. (1978) Pathological evaluation of paratuberculosis in naturally infected cattle. *Veterinary Pathology* **15**, 196-207

DUKES, T. W., GLOVER, G. J., BROOKS, B. W., DUNCAN, J. R. & SWENDROWSKI, M. (1992) Paratuberculosis in saiga antelope (*Saiga tatarica*) and experimental transmission to domestic sheep. *Journal of Wildlife Diseases* **28**, 161-170

GREIG, A., STEVENSON, K., HENDERSON, D., PEREZ, V., HUGHES, V., PAVLIK, I., HINES, M. E., McKENDRICK, I. & SHARP, J. M. (1999) Epidemiological study of paratuberculosis in wild rabbits in Scotland. *Journal of Clinical Microbiology* **37**, 1746-1751

GREIG, A., STEVENSON, K., PEREZ, V., PIRIE, A. A., GRANT, J. M. & SHARP, J. M. (1997) Paratuberculosis in wild rabbits (*Oryctolagus cuniculus*). *Veterinary Record* **140**, 141-143

HARRIS, S. & LLOYD, H. G. (1991) Fox, *Vulpes vulpes*. In A Handbook of British Mammals. 3rd edn. Eds G. B. Corbet, S. Harris. Oxford, Blackwell Scientific. pp 351-367

KING, C. M. (1991) Stoat, *Mustela erminea*. In A Handbook of British Mammals. 3rd edn. Eds G. B. Corbet, S. Harris. Oxford, Blackwell Scientific. pp 377-387

McCLURE, H. M., CHIODINI, R. J., ANDERSON, D. C., SWENSON, R. B., THAYER, W. R. & COUTU, J. A. (1987) *Mycobacterium paratuberculosis* infection in a colony of stumptail macaques (*Macaca arctoides*). *Journal of Infectious Disease* **155**, 1011-1019

PEREZ, V., GARCIA-MARIN, J. F. & BADIOLA, J. J. (1996) Description and classification of different types of lesion associated with natural paratuberculosis infection in sheep. *Journal of Comparative Pathology* **114**, 107-122

RIEMANN, H., ZAMAN, M. R., RUPPANNER, R., AALUND, O., JORGENSEN, J. B., WORSAAE, H. & BEHYMER, D. (1979) Paratuberculosis in cattle and free-living exotic deer. *Journal of the American Veterinary Medical Association* **174**, 841-843

SHARP, J. M., STEVENSON, K., CHALLANS, J. A., RAMAGE, C., HITCHCOCK, D. & REID, H. (1996) Mycobacterial infections of free-living deer in Scotland. Proceedings of the Fifth International Colloquium on Paratuberculosis, September 29-October 4, 1996, Madison, Wisconsin, USA, a meeting of the International Association for Paratuberculosis. Eds R. J. Chiodini, M. E. Hines, M. T. Collins. East Providence, Evergreen Press. pp 180-182

WILLIAMS, E. S., SNYDER, S. P. & MARTIN, K. L. (1983) Pathology of spontaneous and experimental infection of North American wild ruminants with *Mycobacterium paratuberculosis*. *Veterinary Pathology* **20**, 274-291

Monitoring ileitis and *Lawsonia intracellularis* in abattoir pigs

T. K. JENSEN, K. MØLLER, G. CHRISTENSEN, T. D. LESER, S. E. JORSAL

Veterinary Record (1999) **145,** 613-615

T. K. Jensen, DVM, PhD,
M. Møller, DVM, PhD,
T. D. Leser, PhD,
S. E. Jorsal, DVM, PhD,
Danish Veterinary Laboratory, Bülowsvej 27, DK-1790 Copenhagen V, Denmark
G. Christensen, DVM,
Federation of Danish Pig Producers and Slaughterhouses, DK-8620 Kjellerup, Denmark

PORCINE proliferative enteropathy (PE) is a transient intestinal disease affecting the mucosa in the aboral part of the small intestine, particularly the ileum. The disease is characterised by adenomatous proliferation of epithelial cells infected by the obligate intracellular bacterium *Lawsonia intracellularis*, and is grossly seen as a thickening of intestinal mucosa (Rowland and Lawson 1992, McOrist and others 1995). Until recently, the diagnosis was achieved histologically by the demonstration of typical lesions in the mucosa and the presence of intracellular bacteria in epithelial cells, thus requiring a dead or euthanased pig before an accurate diagnosis could be made. For this reason, macroscopic examination of intestines at slaughter has been used to monitor the disease, but the value of the method as a diagnostic test has varied (Rowland and Hutchings 1978, Pointon 1989, Christensen and Cullinane 1990, Jones and others 1993).

FIG 1: Cross-section of ileum without abnormalities, grade 0 (left) and cross section of a rigid ileum graded 2 by palpation (right). The grade 2 ileum shows evident thickening of the muscular wall (arrow)

The aim of this study was to evaluate the visual and palpatory demonstration of increased ileum thickness at slaughter in the monitoring of ileitis and PE. To prevent faecal contamination of the surroundings at the abattoir, the monitoring was performed without making incisions in the intestines. The visual and palpatory observations were compared with the histopathological examination and detection of *L intracellularis* by immunohistochemistry and polymerase chain reaction (PCR).

Intestines from 25 pigs with visual and palpatory thickening of the ileum were selected, together with 14 intestines without abnormalities at postmortem examination at a Danish abattoir. A veterinarian graded the intestines into four groups according to the degree of thickening and rigidity of the intestines by palpation: grade 0 = no abnormalities detected; grade 1 = slightly increased thickness and rigidity of the intestinal wall; grade 2 = noticeably increased rigidity and thickness (up to 4 mm) of the intestinal wall, hyperaemia and slight oedema of the ileal serosa; and grade 3 = extreme thickening (>4 mm) of the intestinal wall and evident oedema of the ileal serosa. The entire ileum was collected from each pig, together with a faeces sample from the colon descendens. The ileal mucosa was examined for gross lesions 24 hours after slaughter in order to reverse muscular contractions before the ileum was divided into two samples. One sample and the faeces sample were processed for PCR as described by Møller and others (1998), whereas a 2 cm cross-section of the other sample was fixed in 10 per cent neutral buffered formalin and processed routinely for histology; one section was stained using haematoxylin and eosin and another section was stained immunohistochemically using an indirect immunofluorescence (IMF) procedure (Jensen and others 1997) with monoclonal antibodies to *L intracellularis* (McOrist and others 1987). The microscopical examination was performed by a veterinarian unaware of the gross grading. In 10 pigs, PCR and immunofluorescence tests were performed twice on the same samples.

The grading, histopathological and microbiological findings of the 25 intestines with visual and palpatory thickening of the ileum is shown in Table 1, together with results for the 14 intestines without gross abnormalities. The classification of the intestines was as follows: there were 14 grade 0, nine grade 1, and 15 grade 2 intestines, and one grade 3 intestine. The cross-section of a grade 0 ileum and a grade 2 ileum with evident thickening of the muscular wall are shown in Fig 1. Macroscopically, mucosal changes consistent with PE were not observed in any of the pigs, but some of the intestines

ELSEVIER

Veterinary Immunology and Immunopathology
77 (2000) 311–319

Veterinary immunology and immunopathology

www.elsevier.com/locate/vetimm

Short communication

Modulation of γδ T cells and CD1 in *Mycobacterium avium* subsp. *paratuberculosis* infection

P.M. Beard[a,*], S.M. Rhind[b], M.C. Sinclair[c], L.A. Wildblood[a], K. Stevenson[a], I.J. McKendrick[d], J.M. Sharp[a], D.G. Jones[a]

[a]*Moredun Research Institute, International Research Centre, Pentlands Science Park, Bush Loan, Penicuik, Midlothian, Scotland EH26 0PZ, UK*
[b]*Department of Veterinary Pathology, Easter Bush Veterinary Centre, University of Edinburgh, Bush Loan, Midlothian, Scotland EH26 0PZ, UK*
[c]*Department of Veterinary Pathology, University of Edinburgh, Summerhall, Edinburgh, Scotland EH9 1QM, UK*
[d]*Biomathematics and Statistics Scotland, The King's Buildings, Edinburgh, Scotland EH9 3JZ, UK*

Received 30 March 2000; received in revised form 14 August 2000; accepted 24 August 2000

Abstract

M.a. paratuberculosis is the causal agent of paratuberculosis (Johne's disease). Recent work has suggested that γδ T cells may play an important role in the early immunological response to mycobacterial diseases, and that CD1 may act as a non-classical MHC molecule in antigen presentation to these γδ T cells. Experimental infection of neonatal lambs with *M.a. paratuberculosis* was used to investigate the changes in γδ T cells and CD1 molecules in the gut associated lymphoid tissue 4 weeks after inoculation. Immunohistochemistry was used to label the γδ lymphocytes and CD1 molecules. An increase in the number of γδ T cells was noted in both the jejunal and ileal Peyer's patches in the gut of infected lambs, but no statistically significant change was found in the mesenteric lymph nodes. There were no obvious changes in the CD1 molecules in any tissue. This work suggests that γδ T cells may play a role in the initial immunological events of paratuberculosis infection.

Keywords: Mycobacteria; Paratuberculosis; γδ lymphocytes; CD1; Peyer's patches; Ruminant

* Corresponding author. Tel.: +44-131-445-5111; fax: +44-131-445-6111.
E-mail address: bearp@mri.sari.ac.uk (P.M. Beard).

0165-2427/00/$ – see front matter
PII: S0165-2427(00)00238-5

1. Introduction

Paratuberculosis, or Johne's disease, is a chronic enteritis affecting both wild and domestic ruminants, caused by *Mycobacterium avium* subsp. *paratuberculosis* (*M.a. paratuberculosis*). While much of the epidemiology and pathogenesis of the disease has yet to be elucidated, it is thought that infection is most commonly established in the first few weeks of life (Chiodini et al., 1984). A proportion of infected animals quickly expel the organism and recover completely, while others enter a long period of subclinical infection, with small numbers of *M.a. paratuberculosis* residing in the macrophages of the gut associated lymphoid tissues (Clarke, 1997). After a number of years, a proportion of these subclinically affected animals develop clinical disease — *M.a. paratuberculosis* multiplies in the intestines, resulting in enteritis, and associated clinical signs of weight loss, diarrhoea, and eventually death. While much work on paratuberculosis has focused on the balance between late subclinical and clinical disease, little work has been done to investigate events during the earliest stages of infection.

The earliest pathological changes found in paratuberculosis have been identified in the jejunal Peyer's patch (JPP), ileal Peyer's patch (IPP), and mesenteric lymph node (MLN) (Nisbet et al., 1962; Perez et al., 1996). Studies have linked *M.a. paratuberculosis* with M cell endocytosis, suggesting that the organism utilises the M cells in the follicle associated epithelium overlying the JPP and IPP as a method to breach the gut epithelium barrier, and establish infection (Momotani et al., 1988; Fujimura and Owen, 1996). Thus the earliest, and possibly most vital, interactions between the host immune system and *M.a. paratuberculosis,* occur in these gut associated lymphoid areas (GALT).

While most T cells express the $\alpha\beta$ T cell receptor (TCR), this report has concentrated on the smaller group that expresses a $\gamma\delta$ TCR. $\gamma\delta$ T cells are found in relatively high numbers at epithelial surfaces, including the gut mucosa, and have been found to increase both in vivo and in vitro in response to early mycobacterial infection (Janis et al., 1989; reviewed by Boom, 1999). Ruminants have a much higher percentage of $\gamma\delta$ T cells when compared to humans or mice — the number of $\gamma\delta$ T cells in young ruminants can be up to 50% of all T cells present, whereas $\gamma\delta$ T cells comprise only 1–5% of the total circulating lymphocyte population in humans and mice (Evans et al., 1994). Taken together, these observations suggest that, in young ruminants, $\gamma\delta$ T cells may be particularly important in the defence of mucosal surfaces against mycobacterial infections. $\gamma\delta$ T cells in all species generally do not express either CD4 or CD8 molecules, and are consequently considered as MHC class unrestricted. Suggested alternative antigen presenting mechanisms include CD1 molecules, which are known to present glycolipids derived from mycobacteria (Sieling et al., 1999). The aim of this study was to investigate alterations in the $\gamma\delta$ T cell population and expression of CD1 in relevant lymphoid areas in the gut during the early stages of *M.a. paratuberculosis* infection.

2. Materials and methods

The experimental model used was based on an earlier study (Begara-McGorum et al., 1998). The inoculation regime and determination of the end point of the current

experiment was derived from the results of the earlier study. Ten lambs (Suffolk–Texel crosses) of either sex, aged between 8 and 19 days old, were randomly allocated to two equal groups — group 1 served as the control group, and group 2 as the infected group. The dams of the lambs were negative for *M.a. paratuberculosis* infection as judged by ELISA. Lambs in group 2 were each dosed orally with approximately 1×10^9 colony forming units (cfu) of *M.a. paratuberculosis* (JD88/107) suspended in 5 ml of sterile phosphate buffered saline (PBS) on days 0, 2 and 4. Group 1 received 5 ml of sterile PBS on the same days. The lambs were euthanised with intravenous barbiturate solution 28 days after the first inoculation. All experimental procedures and management protocols were passed by the Moredun Research Institute Ethics Committee, and carried out under approved British Home Office licences in accordance with the Animals (Scientific Procedures) Act 1986.

2.1. Culture

Samples of MLN and IPP were collected aseptically, and cultured on Middlebrook 7H11 medium supplemented with Selectatabs (MAST Laboratories Ltd., Merseyside, UK), OADC enrichment medium (Difco, Surrey, UK), and mycobactin J (Allied Monitor, Fayteet, MO) as described previously (Greig et al., 1997).

2.2. Histopathology

At necropsy, tissues were inspected grossly, and samples of JPP, IPP and MLN were collected for routine histopathological analysis. The samples were fixed in 10% buffered formol saline for a minimum of 24 h, trimmed, dehydrated through graded alcohols, embedded in paraffin wax and 5 μm-thick sections cut. These were stained with haematoxylin and eosin for routine examination, and for acid fast bacilli (AFB) by the Ziehl–Neelsen method.

2.3. Immunohistochemistry

Samples of JPP, IPP, and MLN were collected at necropsy, snap frozen in a slurry of dry ice and isopentane for approximately 30 s and stored at −70°C until required. Cryostat sections, 6 μm thick were cut from the frozen tissues onto either vectabond® treated (Vector Labs, Peterborough, UK) or superfrost® plus (BDH Merck, Leistershire, UK) slides, air dried overnight, fixed for 10 min in acetone, and stored at −20°C. Labelling was carried out by an indirect immunoperoxidase technique using a commercial kit (Vectastain Elite ABC kit, Vector Labs), following the manufacturers instructions. Slides were rehydrated for 10 min in a blocking buffer (2% normal sheep serum in PBS), and then incubated in 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Incubation with primary antibody was carried out for 1 h at room temperature. A negative control (no primary antibody) was included each time. Visualisation of bound antibody–HRP complex was performed using a DAB substrate kit (Vector Labs). Following colour development (2–5 min) sections were washed in tap water, lightly counterstained in haematoxylin, dehydrated through graded alcohols and mounted in DePeX mounting medium (BDH Merck).

γδ T cells were labelled with monoclonal antibody (mAb) IAH-CC15 which recognises the surface antigen WC1 (Wijngaard et al., 1992; Crocker et al., 1993) expressed on γδ T cells which lack CD2, CD4 and CD8. CD1 molecules were labelled with mAb IAH-CC20 (Howard et al., 1993) which has a CD1b-like pattern of reactivity, belonging to the cluster BoCD1w2 (Howard and Naessens, 1993).

After labelling with either CC15 or CC20, the sections were examined microscopically, and the number of positively labelled cells counted. Briefly, the section was divided into four areas: villous, dome, interfollicular, and follicular. The number of positive cells per approximately 100 total cells was calculated in at least two separate locations for each area on each slide. Thus for each animal, a minimum of eight counts was obtained for each tissue, and each antibody. MLN were examined under 400× magnification, and the number of positive cells per high power field was counted. At least five high powered fields were counted in both the cortex and medulla.

Samples of JPP, IPP, and MLN were collected for fluorescent antibody cell sorting (FACS) analysis, to investigate changes in other inflammatory cell subsets. These results will be reported in a later paper.

2.4. Statistics

It was necessary to use generalised linear mixed models (GLMM), rather than simpler statistical analyses, because of the non-independence of counts drawn from the same animal (Brown and Prescott, 1999). For data from JPP and IPP, a separate analysis was carried out for each combination of tissue, antibody, and area. The data were the number of positive cells from the total number of cells observed during each count. Accordingly, in each analysis, a GLMM with binomial variation and a logit link function was fitted, modelling between sheep and residual variation as random effects and infective status as a fixed effect. Tests of significance were carried out on the logit scale.

For data from the MLN tissue the data were numbers of positive cells. A GLMM with Poisson variation and a logarithmic link function was fitted, again modelling between sheep and residual variation as random effects and infective status as a fixed effect. Tests of significance were carried out on the logarithmic scale.

3. Results

3.1. Culture and histopathology

No gross lesions were evident at necropsy, and no microscopic lesions consistent with *M.a. paratuberculosis* infection were noted on routine histopathology. No AFB were identified in any tissues. No bacteria were grown from any tissue sample.

3.2. Immunohistochemistry

γδ T cells and CD1 molecules were seen in the villi, dome, and interfollicular areas of the gut (see Fig. 1), as well as the cortex and medulla of the MLN. However, very few γδ

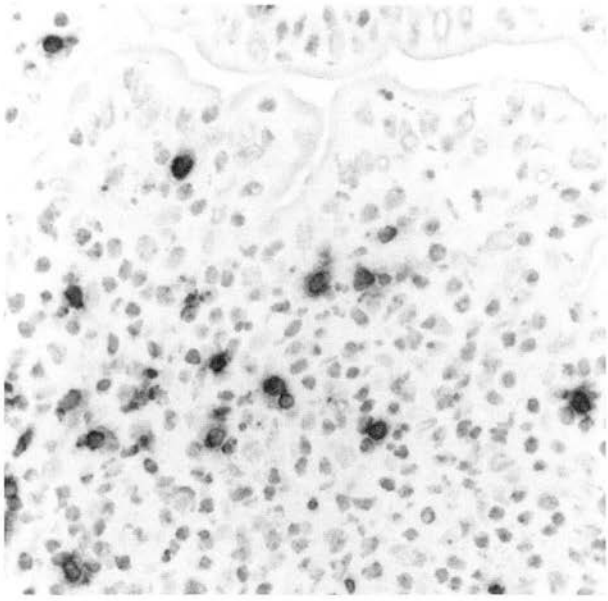


Fig. 1. Positively labelled γδ T cells in a dome area of the JPP. Magnified 300×.

T cells and CD1 molecules were seen in the follicular areas in both the JPP and IPP, consequently, this data was not included in the statistical analysis. Similarly, CD1 molecules were very rarely observed in the villi of IPP in both control and infected tissue, and were also excluded from statistical analysis. On examination of the frozen tissues, samples of the JPP were found to be missing from one control and one infected lamb. Consequently, the data for the JPP is calculated from eight animals.

3.3. Statistics

The results of the statistical analysis are summarised in Tables 1 and 2. Comparisons of averages from the raw data are not useful, because of potential bias from the unequal numbers of counts being made from each animal. Instead, the GLMM fits a statistical model for the probability of each observed cell being positive. The results of these models can be "back-transformed" to provide estimates for the average percentage of positive cells observed (in IPP and JPP tissues) or the average number of positive counts (MLN data) in both infected and uninfected animals. The GLMM also provides an estimate of the standard error of the difference between these averages on the transformed scale (either logit or logarithmic, as appropriate), from which a p-value can be calculated. The Satterthwaite approximation was used to derive the appropriate degrees of freedom for use in these tests.

There was an increase in the numbers of γδ T cells in the infected group in all three areas (villi, dome and interfollicular) of the JPP and IPP. Using the GLMM model, it was

Table 1
Summary of GLMM statistical analysis of JPP and IPP, showing the estimated mean percentage of cells counted as positive in uninfected and infected groups, the standard error of the difference between these percentages and the corresponding p-values

Ab	Tissue	Area	Uninfected group[a]	Infected group[a]	S.E. of difference[b]	p-Value
γδ	JPP	Villi	5.4	7.0	0.256	0.33
		Dome	3.2	6.9	0.256	0.03*,**
		Interfollicular	2.7	4.7	0.259	0.08**
	IPP	Villi	5.3	9.2	0.218	0.03*,**
		Dome	2.9	5.7	0.320	0.06**
		Interfollicular	2.2	4.0	0.322	0.10**
CD1	JPP	Villi	0.1	0.2	1.814	0.62
		Dome	5.4	8.9	0.446	0.28
		Interfollicular	5.4	3.5	0.278	0.17
	IPP	Villi	NA	NA	NA	NA[c]
		Dome	2.6	2.8	0.337	0.89
		Interfollicular	3.8	5.8	0.327	0.22

[a] Estimated mean percentage of positively stained cells.
[b] On logit scale.
[c] Model failed to converge, due to lack of differences between groups.
* $p < 0.05$; ** $p < 0.10$.

found that, with the exception of the villous area of the JPP, these increase were either statistically significant at the 5% level, or were close to such significance ($p < 0.1$). The γδ T cell numbers were higher in the cortex and medulla of the MLN from the infected group, but these differences were not statistically significant.

There were no significant differences in the numbers of CD1 molecules between the groups, in any area of the JPP, IPP, or MLN. There was a small decrease in the number of CD1 cells in the interfollicular area of the JPP in infected animals, but this difference was not statistically significant.

Table 2
Summary of GLMM statistical analysis of MLN, showing the estimated mean count per high powered field of positive cells in uninfected and infected groups, the standard error of the difference between these counts and the corresponding p-values

Ab	Tissue	Area	Uninfected group[a]	Infected group[a]	S.E. of difference[b]	p-Value
γδ	MLN	Cortex	27.3	45.9	0.393	0.23
		Medulla	22.8	36.5	0.295	0.15
CD1	MLN	Cortex	9.1	15.7	0.493	0.30
		Medulla	3.4	3.4	0.342	0.94

[a] Estimated mean count of positively stained cells per high powered field.
[b] On logarithmic scale.

4. Discussion

This experiment investigated changes in the γδ T cell population and CD1 expression in young lambs in response to exposure to *M.a. paratuberculosis*. The specific areas thought to be involved in early interactions with the *M.a. paratuberculosis* organisms, i.e. the gut associated lymphoid tissues (JPP, IPP and MLN) were examined with respect to expression of WC1 and CD1b surface markers using monoclonal antibodies. The changes noted in other inflammatory cell subsets will be reported in a later communication.

The experimental model used was based on earlier studies (Begara-McGorum et al., 1998) which reported the presence of *M.a. paratuberculosis* lesions in, and growth of *M.a. paratuberculosis* from, the GALT. The most suitable end point of the experiment was estimated from these earlier studies to be 28 days post-inoculation.

The lack of pathological changes in the lymphoid regions of the gut in this experiment was not unexpected. Although earlier work had suggested that the inoculation regime would produce evidence of infection (Begara-McGorum et al., 1998), other work has shown early changes in *M.a. paratuberculosis* infection may be subtle or even absent (Nisbet et al., 1962; Juste et al., 1994; Sigurdardottir et al., 1999). The absence of any positive cultures may be attributable to the low number of organisms thought to be in the tissues at this stage of infection. Although these observations may reflect the extended incubation period of paratuberculosis and the subtlety of the early pathology of the disease, it remains a possibility that infection in this experimental model was not established. Thus, the immunological changes described could be merely a response to *M.a. paratuberculosis* challenge rather than changes seen in early *M.a. paratuberculosis* infection.

The increase found in the number of γδ T cells in the JPP, IPP and MLN, in response to *M.a. paratuberculosis* exposure, suggests that these cells may be an important component of the early host immune response to *M.a. paratuberculosis* infection. However, the exact role of the γδ T cells remains unknown — whether, in these specialised lymphoid compartments, they assist the host to combat the infection, or whether they suppress other host immune responses, and thus aid *M.a. paratuberculosis* to evade the immune system and achieve a persistent state in the lymphoid tissue, is unclear.

Most of our knowledge of γδ T cells comes from human and murine studies. γδ T cells have been shown to increase in response to a number of intracellular pathogens including mycobacteria (reviewed by Boismenu and Havran, 1998). The most common hypothesis is that γδ T cells bridge the gap between early innate immune mechanisms, and the later more complex and specific αβ T cell immune responses. γδ T cells are most populous at epithelial surfaces, including skin, gut and airways, supporting theories of their role as first line defence against invading pathogens.

There have been few studies reported specifically on ruminant γδ T cells in mycobacterial infection. Pollock et al. (1996) examined peripheral blood levels of γδ T cells in calves experimentally infected with *M. bovis*. They reported that γδ T cells initially decreased after infection, and then increased, suggesting localisation to developing lesions, followed by clonal expansion. Our results would support this general premise. Cross et al. (1996) failed to find evidence of increased activity of γδ T cells in red deer 8 weeks after experimental infection with *M. bovis*, but suggested that this was due to the length of time elapsed between infection and sampling.

One factor crucial in the elucidation of the role of the increased numbers of γδ T cells in response to *M.a. paratuberculosis* exposure is the identity of the antigen presenting molecule (APM) involved in the γδ T cell–*M.a. paratuberculosis* interaction. We found no evidence that CD1 expressing antigen presenting cells were up or down regulated by *M.a. paratuberculosis* exposure, and thus no proof that they do act as APM for γδ T cells. One interesting result, however, was a decrease in the number of CD1 in the interfollicular area of the JPP in the infected group. CD1 binds lipid and glycolipid antigen, for presentation to a wide variety of T lymphocytes, including γδ T cells (Sugita et al., 1998; Porcelli et al., 1989). Modulation of CD1 expression has been described in association with several mycobacterial infections. The best example of this in human pathology is in leprosy where there is upregulation of group 1 CD1 molecules in the 'tuberculoid' form of the disease which is characterised by a strong cell mediated immune response (Sieling et al., 1999). Conversely, infection of antigen presenting cells with *M. tuberculosis* has been shown to downregulate the expression of human CD1b (Stenger et al., 1998). This observation is consistent with an immune evasion mechanism. Although the decrease observed in the number of CD1 cells in the JPP of the infected group did not reach statistical significance, it may represent a mild in vivo illustration of the down regulation mechanism employed by mycobacteria reported by Stenger et al. (1998).

5. Conclusion

This paper reports preliminary results of investigations into the role of γδ T cells and CD1 molecules in the early immune response to *M.a. paratuberculosis* infection. These results support the hypothesis that γδ T cells play a role in early mycobacterial infections, and suggest that events in JPP and IPP may have important implications for the outcome of *M.a. paratuberculosis* infection. Further work is needed to elucidate the role of CD1 as an APM for γδ T cells. The experimental model described has potential for investigating this and other unexplained features of γδ T cells in the context of mycobacterial infection.

Acknowledgements

The authors would like to thank the following for their invaluable contribution to the above work: Jock McCracken and Iris Campbell for assistance with the animal work, Amanda Pirie and Karen Rudge for bacterial cultures, Neil MacIntyre, Andrew Dawson and Sharon Moss for technical help, and Dave Buxton for advice and support. This work was supported by grants from the Biotechnology and Biological Sciences Research Council (BBSRC) and the Scottish Executive for Rural Affairs Department (SERAD).

References

Begara-McGorum, I., Wildblood, L.A., Clarke, C.J., Connor, K.M., Stevenson, K., McInnes, C.J., Sharp, J.M., Jones, D.G., 1998. Early immunopathological events in experimental ovine paratuberculosis. Vet. Immunol. Immunopathol. 63, 265–287.

Boismenu, R., Havran, W.L., 1998. γδ T cells in host defence and epithelial cell biology. Clin. Immunol. Immunopathol. 86, 121–133.

Boom, W.H., 1999. γδ T cells and *Mycobacterium tuberculosis*. Microb. Infect. 1, 187–195.

Brown, H., Prescott, R., 1999. Applied Mixed Models in Medicine. Wiley, Chichester, UK.

Chiodini, R.J., Van Kruiningen, H.J., Merkel, R.S., 1984. Ruminant paratuberculosis, Johne's disease; the current status and future prospects. Cornell Vet. 74, 218–262.

Clarke, C.J., 1997. Pathology and pathogenesis of paratuberculosis in ruminants and other species. J. Comp. Path. 116, 217–261.

Crocker, G., Sopp, P., Parsons, K., Davis, W.C., Howard, C.J., 1993. Analysis of the γ/δ T cell restricted antigen WC1. Vet. Immunol. Immunopathol. 39, 137–144.

Cross, M.L., Chinn, N.D., Griffin, J.F.T., Buchan, G.S., 1996. T cell responses to *Mycobacterium bovis* in red deer a large animal model for tuberculosis. Immunol. Cell Biol. 74, 32–37.

Evans, C.W., Lund, B.T., McConnel, I., Bujdoso, R., 1994. Antigen recognition and activation of ovine γδ T cells. Immunology 82, 237–299.

Fujimura, Y., Owen, R.L., 1996. M cells as portals of infection: clinical and pathophysiological aspects. Infect. Agents Dis. 5, 144–156.

Greig, A., Stevenson, K., Perez, V., Pirie, A.A., Grant, J.M., Sharp, J.M., 1997. Paratuberculosis in wild rabbit, *Oryctolagus cuniculus*. Vet. Rec. 140, 141–143.

Howard, C.J., Naessens, J., 1993. Summary of workshop findings for cattle, Tables 1 and 2. Vet. Immunol. Immunopathol. 39, 25–48.

Howard, C.J., Sopp, P., Bembridge, G., Young, J., Parsons, K.R., 1993. Comparison of CD1 monoclonal antibodies on bovine cells and tissues. Vet. Immunol. Immunopathol. 39, 77–83.

Janis, E.M., Kaufmann, S.H.E., Schwartz, R.H., Pardoll, D.M., 1989. Activation of γδ T cells in the primary immune response to *Mycobacterium tuberculosis*. Science 244, 713–716.

Juste, R.A., Garcia Marin, J.F., Peris, B., Saez de Ocariz, C., Badiola, J.J., 1994. Experimental infection of vaccinated and non-vaccinated lambs with *M.a. paratuberculosis*. J. Comp. Path. 110, 185–194.

Momotani, E., Whipple, D.L., Thiermann, A.B., Cheville, N.F., 1988. Role of M cells and macrophages in the entrance of *Mycobacteria paratuberculosis* into domes of ileal Peyer's patches in calves. Vet. Pathol. 63, 255–367.

Nisbet, D.I., Gilmour, N.J.L., Brotherston, J.G., 1962. Quantitative studies of *Mycobacterium johnei* in tissues of sheep. III. Intestinal histopathology. J. Comp. Path. 72, 80–91.

Perez, V., Garcia-Marin, J.F., Badiola, J.J., 1996. Description and classification of different types of lesion associated with natural paratuberculosis infection in sheep. J. Comp. Path. 114, 107–122.

Pollock, J.M., Pollock, D.A., Campbell, D.G., Girvin, R.M., Crockard, A.D., Neill, S.D., Mackie, D.P., 1996. Immunology. 87, 236–241.

Porcelli, S., Brenner, M.B., Greenstein, J.L., Balk, S.P., Terhorst, C., Bleicher, P.A., 1989. Recognition of clusters of differentiation 1 antigens by human $CD4^-CD8^-$ cytolytic T lymphocytes. Nature 341, 447–450.

Sieling, P.A., Julien, D., Dahlem, M., Tedder, T.F., Rea, T.H., Modlin, R.L., Porcelli, S.A., 1999. CD1 expression by dendritic cells in human leprosy lesions: correlation with effective host immunity. J. Immunol. 162, 1851–1858.

Sigurdardottir, O.G., Press, C.McL., Saxegaard, F., Evensen, O., 1999. Bacterial isolation, immunological response, and histopathological lesions during the early subclinical phase of experimental infection of goat kids with *Mycobacterium avium* subspp. *paratuberculosis*. Vet. Pathol. 36, 542–550.

Stenger, S., Niazi, K.R., Modlin, R.L., 1998. Down regulation of CD1 on antigen-presenting cells by infection with *Mycobacterium tuberculosis*. J. Immunol. 161, 3582–3588.

Sugita, M., Moody, D.B., Jackman, R.M., Grant, E.P., Rosat, J.P., Behar, S.M., Peters, P.J., Porcelli, S.A., Brenner, M.B., 1998. CD1 — a new paradigm for antigen presentation and T cell activation. Clin. Immunol. Immunopathol. 87, 8–14.

Wijngaard, P.J.L., Metzelaar, M.J., MacHugh, N.D., Morrison, W.I., Clevers, H.C., 1992. Molecular characterisation of the WC1 antigen expressed specifically on bovine $CD4^-CD8^-$ γδ T lymphocytes. J. Immunol. 149, 3273–3277.

Plate 1,1 A merino sheep with clinical paratuberculosis, exhibiting signs of weight loss (photograph by Kym Abbott)

Plate 1,2 Mucosa of the ileum from a sheep with paratuberculosis, showing corrugations and the yellow discolouration present in some cases of the disease

Plate 1,3 A section of the small intestine from a sheep with paratuberculosis, showing thickened intestine and swollen lymphatics in the mesentry

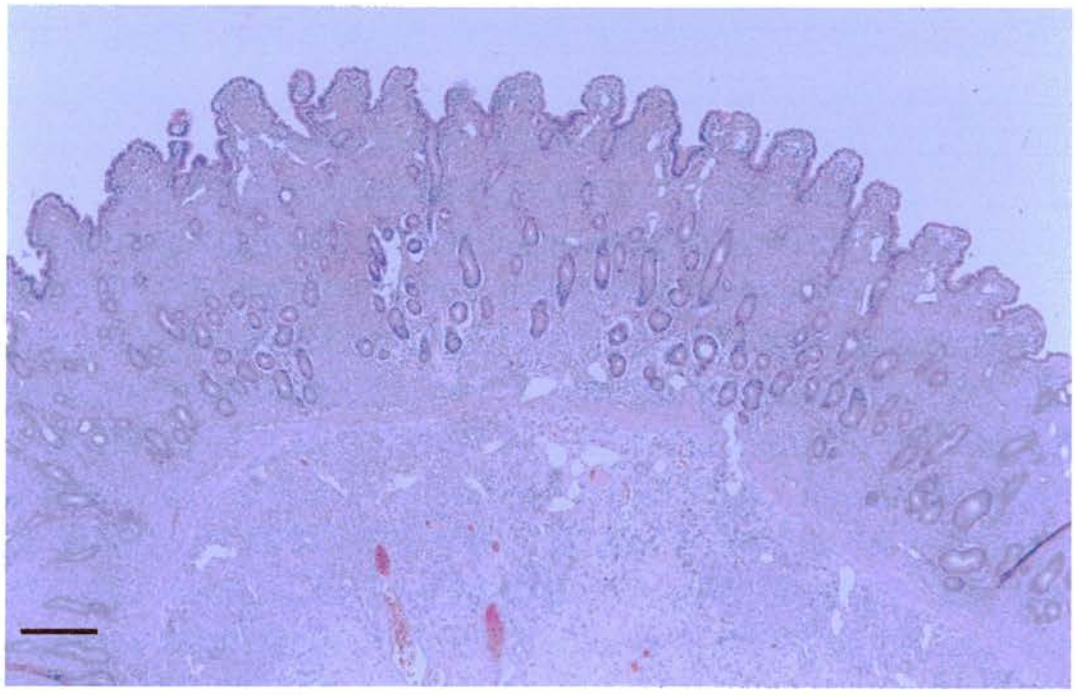


Plate 1,4 Multibacillary paratuberculosis in the terminal ileum of a sheep. The villi are thickened with epithelioid and other chronic inflammatory cells. Changes are also apparent in the submucosa
H&E Bar=125μm

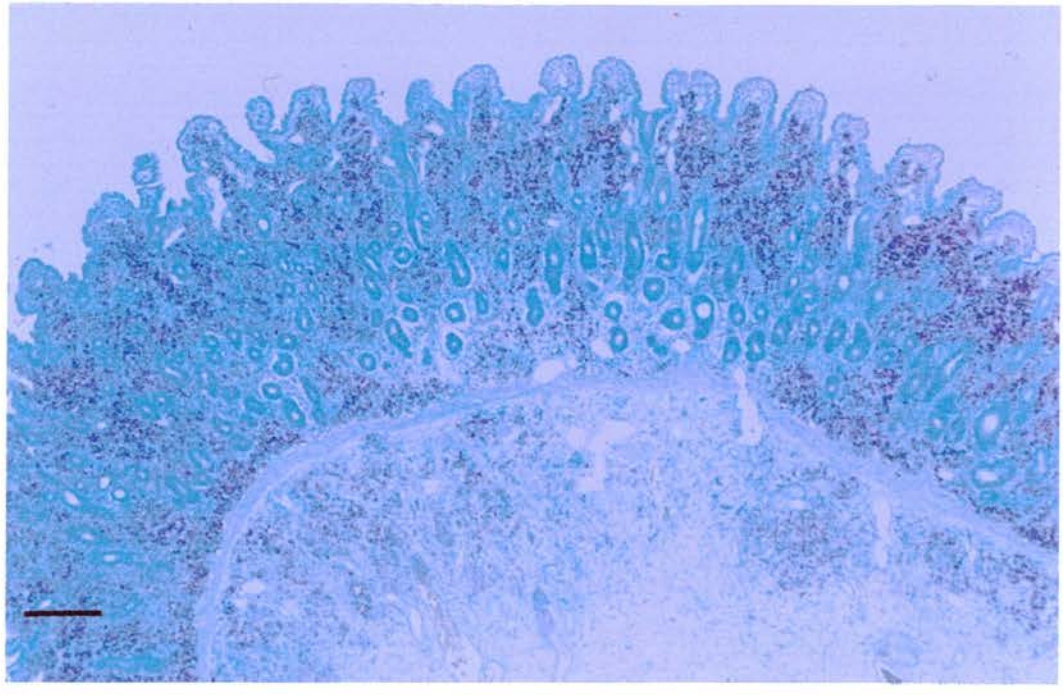


Plate 1,5 Multibacillary paratuberculosis in the terminal ileum of a sheep (serial section to Plate 1,4). Numerous AFB are visible in the villi and submucosa, coloured cherry red.
ZN Bar=125μm

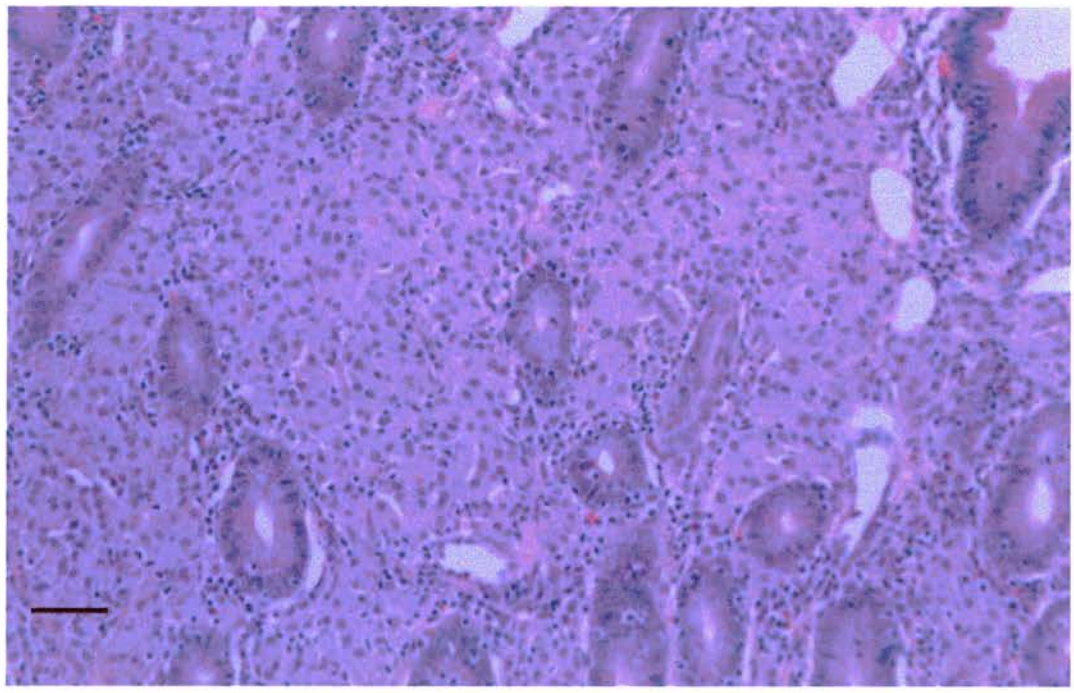


Plate 1,6 A section of villi from the ileum of a sheep with the multibacillary form of paratuberculosis, viewed under high power. Note the numerous large foamy macrophages present.
H&E Bar=50μm

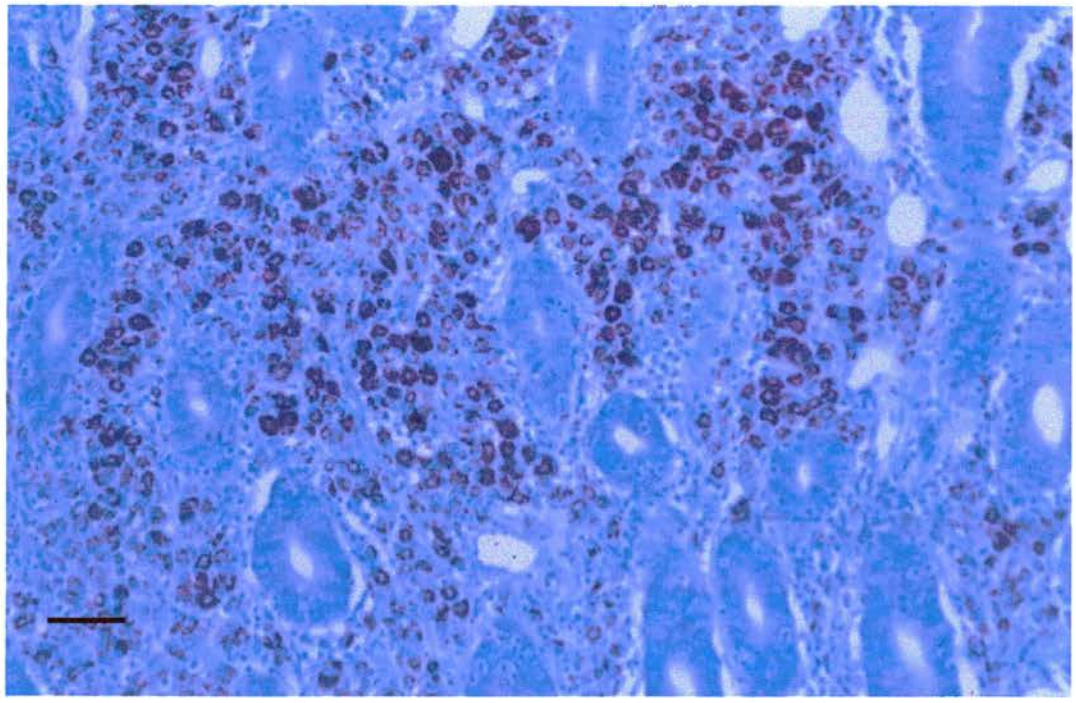


Plate 1,7 Multibacillary paratuberculosis in the ileum of a sheep (serial section of Plate 1,6). Note the numerous AFB in the cytoplasm of the macrophages
ZN Bar=50μm

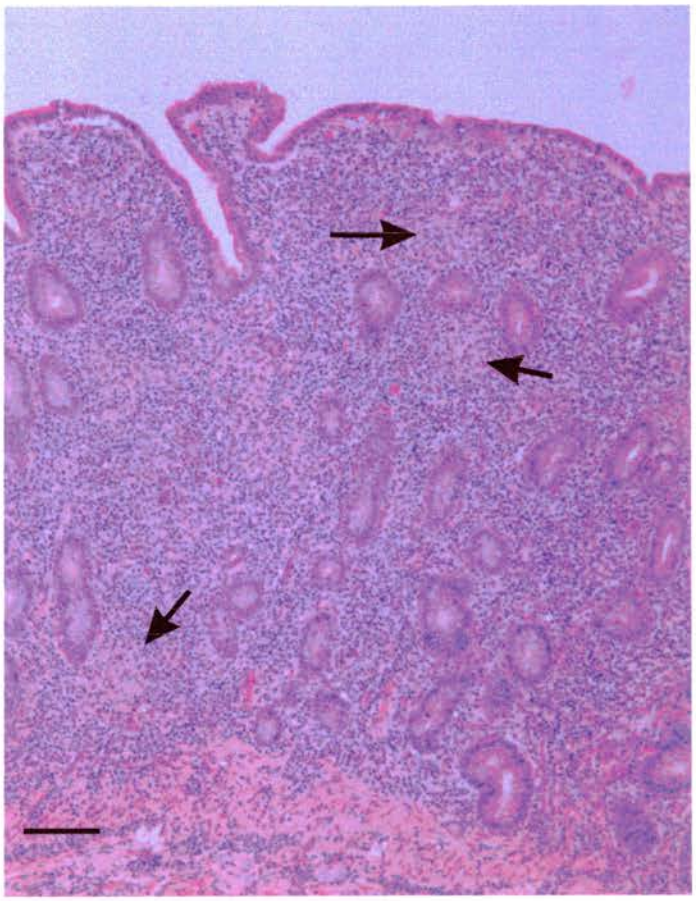


Plate 1,8 A section of ileum from a sheep with paucibacillary paratuberculosis. The villi are fused and stunted, with an associated infiltrate which is predominately lymphocytic with a few admixed macrophages (arrows)
H&E Bar=100μm

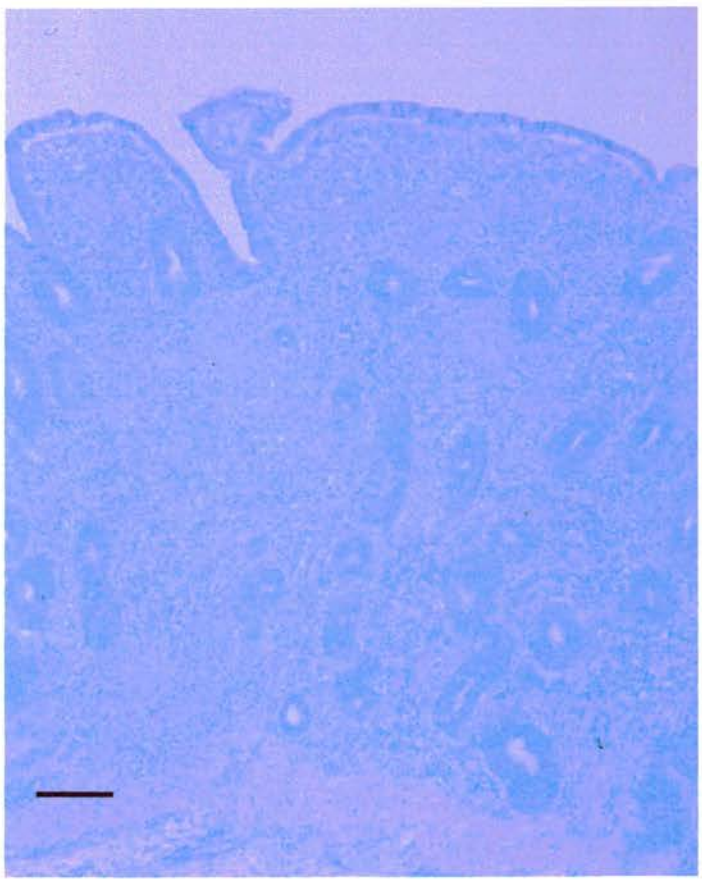


Plate 1,9 Serial section to Plate 1,8 above, with no AFB visible.
ZN Bar=100μm

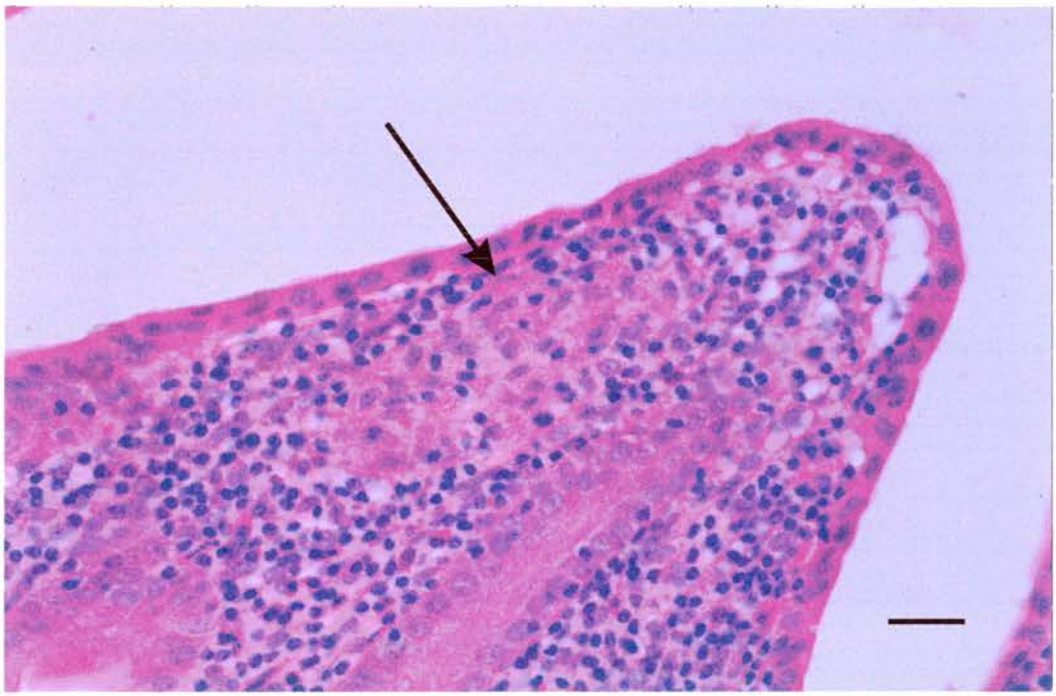


Plate 1,10 The tip of a villus from the ileum of a sheep with paucibacillary paratuberculosis, showing the predominance of lymphocytes, with a small cluster of macrophages (arrow)
H&E Bar=25μm

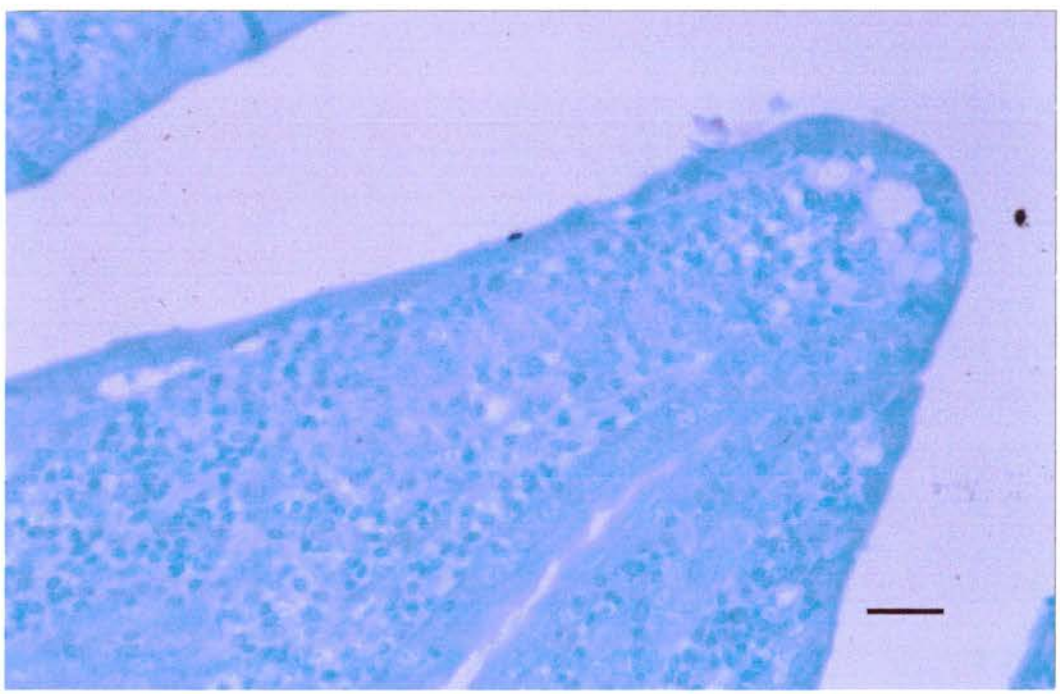


Plate 1,11 Serial section of Plate 1,10 above, demonstrating an absence, in contrast to plate 1,7, of AFB in the cytoplasm of the macrophages.
ZN Bar=100μm

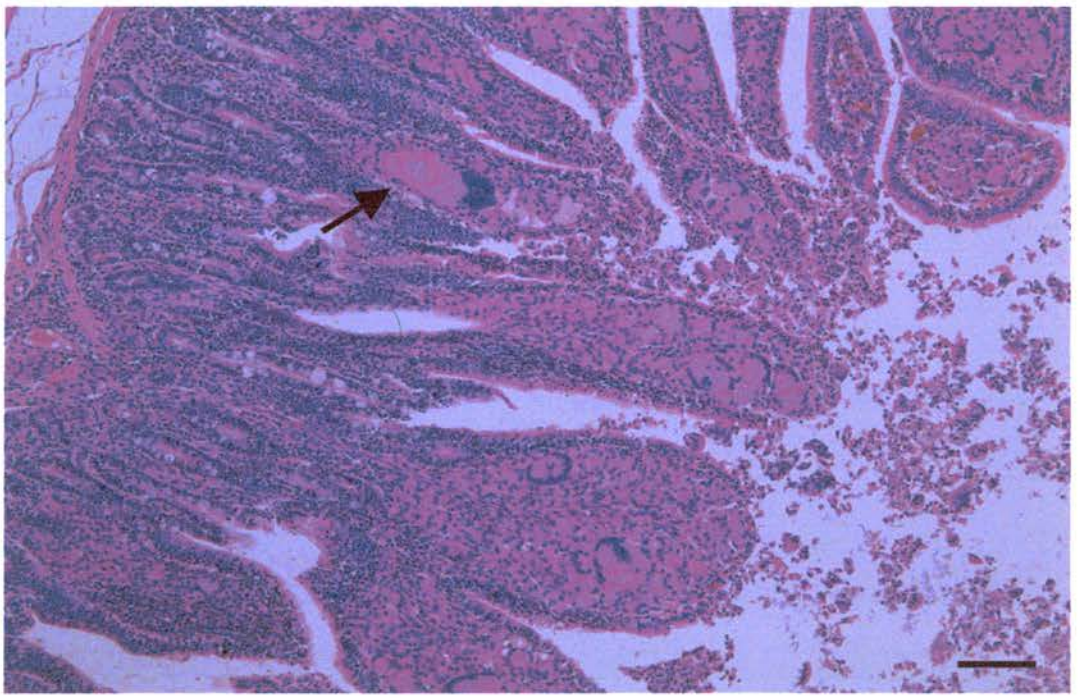


Plate 3,1 A section of the small intestine from a severely affected rabbit, showing markedly thickened "drumstick-shaped" villi, containing numerous epithelioid and giant cells (arrow).
H&E Bar=100μm

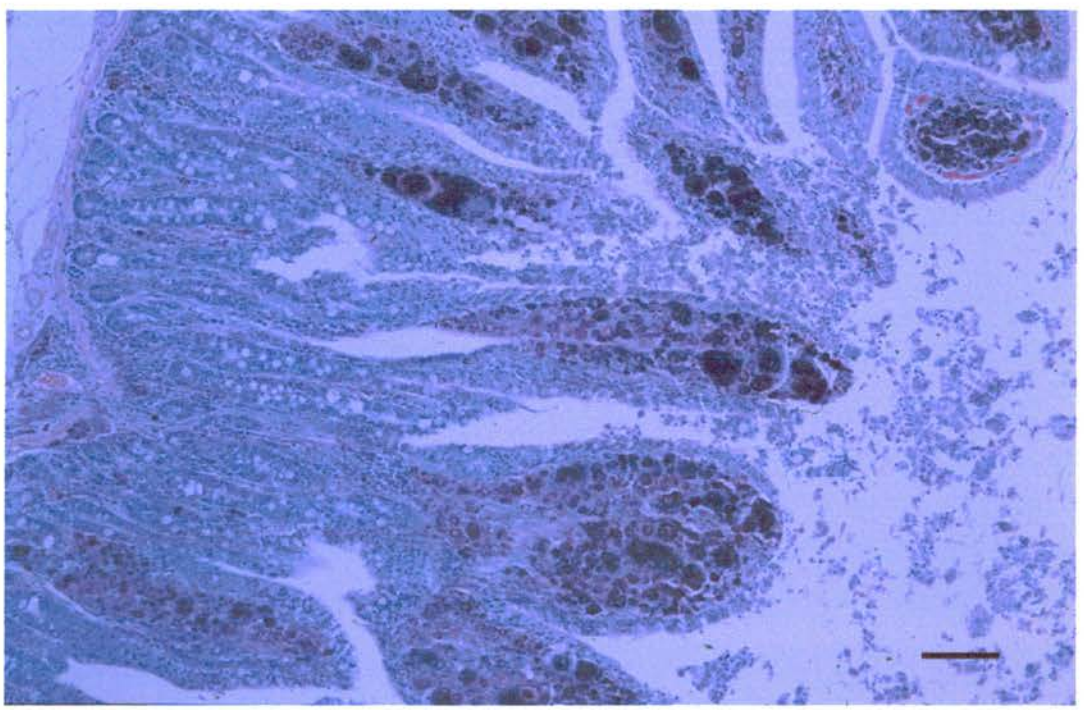


Plate 3,2 Serial section of plate 3,1 above, showing abundant intracellular AFB in the giant and epithelioid cells
ZN Bar=100μm

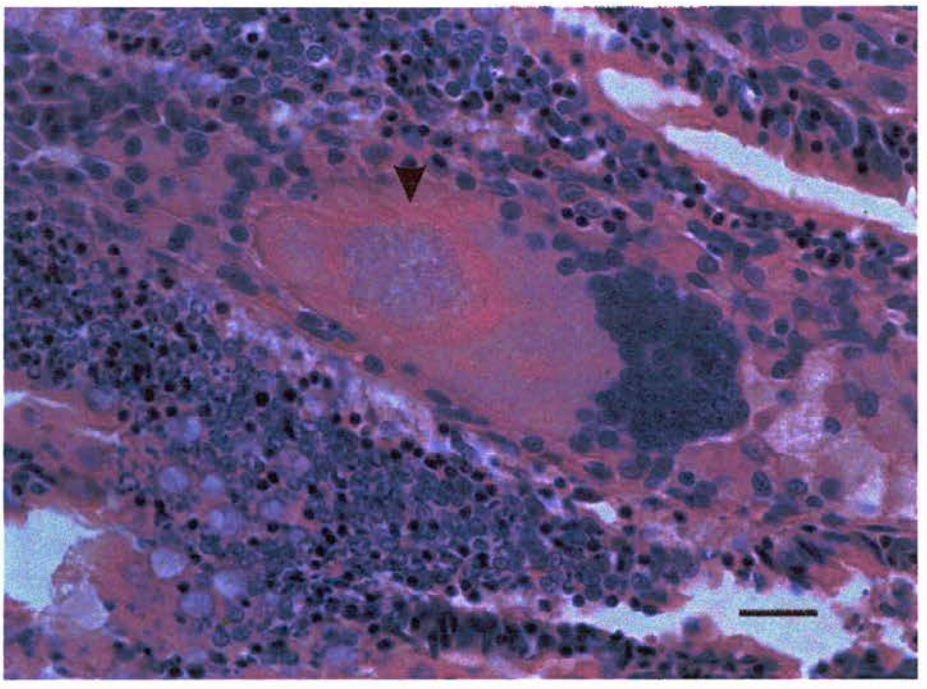


Plate 3,3 Higher magnification of plate 3,1, detailing a prominent giant cell with numerous peripheral nuclei, and a large, pale staining vacuole (arrowhead) in the cytoplasm
H&E Bar=25mm

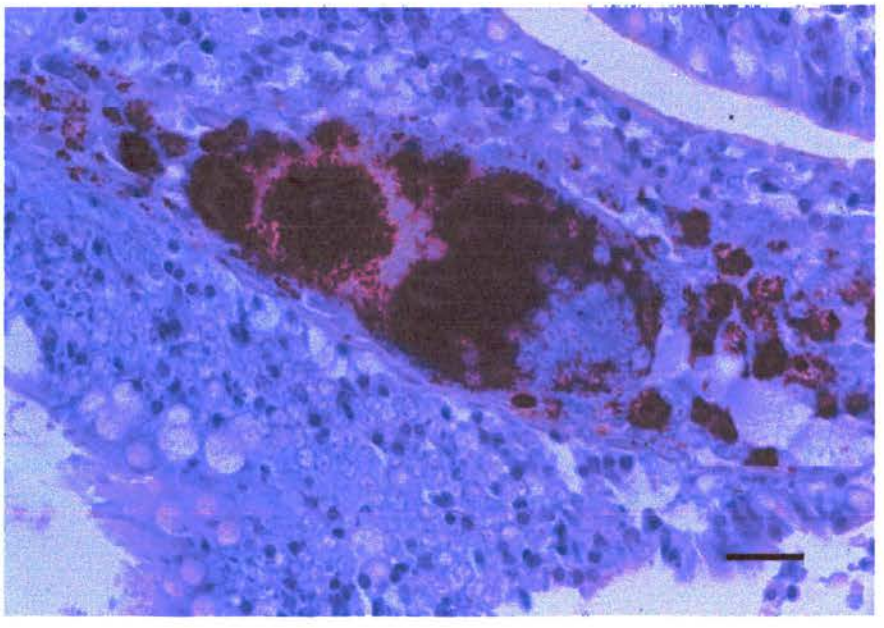


Plate 3,4 Serial section of plate 3,3 above, revealing AFB present in the cytoplasm and vacuole of the giant cell
ZN Bar=25μm

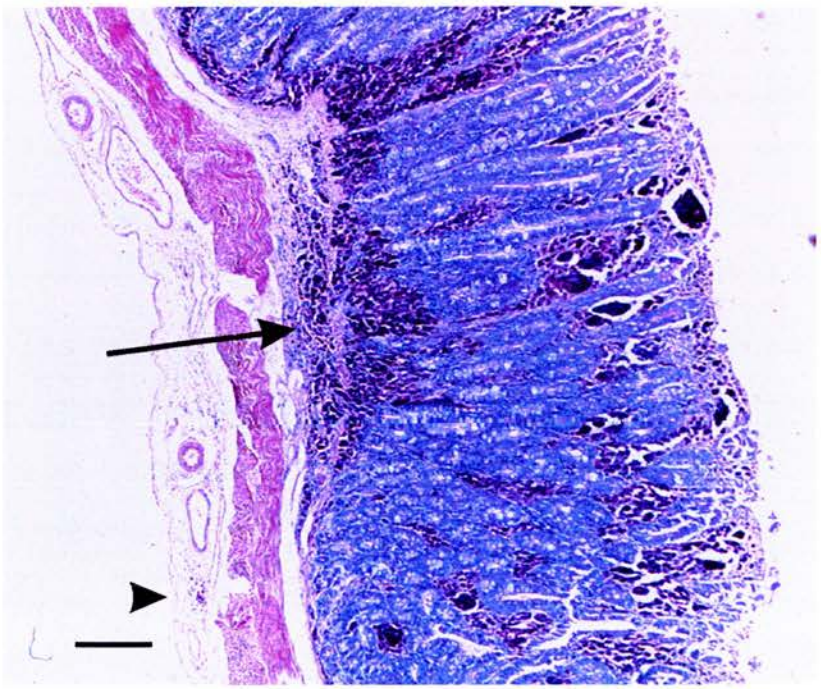


Plate 3,5 A section of small intestine from a severely affected rabbit, with lesions present in the lamina propria, submucosa, and outer serosal layer (arrowhead). The arrow points to a submucosal lymphoid patch with numerous AFB present
ZN Bar=250μm

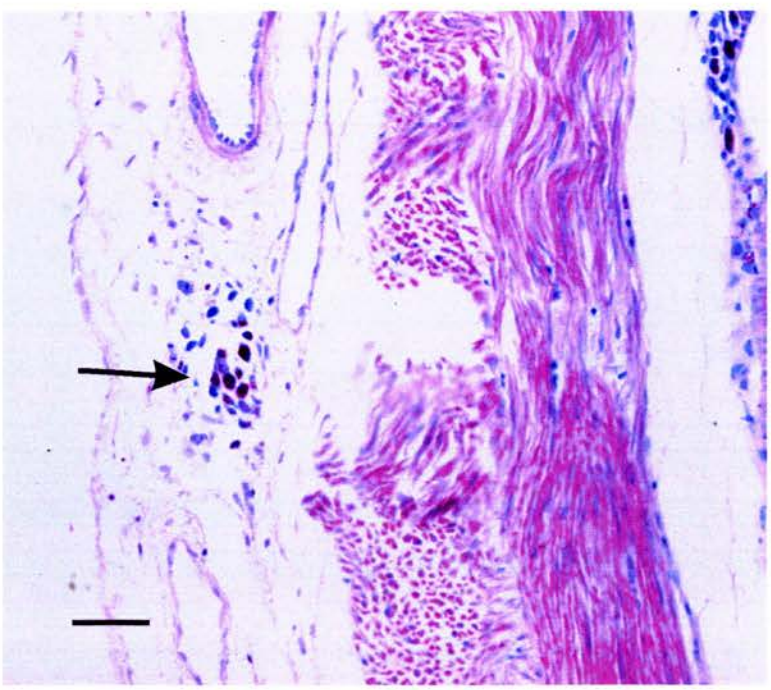


Plate 3,6 Higher magnification of the area near the arrowhead in plate 3,5 above, showing AFB in the cytoplasm of cells in the serosal layer of the intestine (arrow).
ZN Bar=50μm

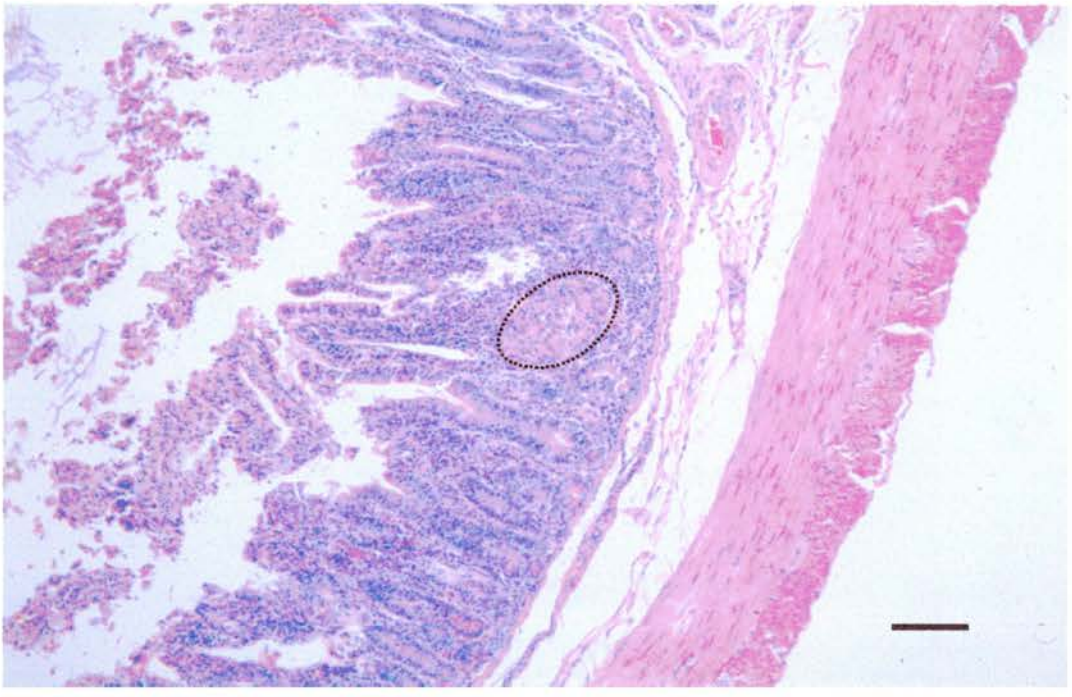


Plate 3,7 A small granuloma (dotted line) is present in the basal area of the lamina propria of the small intestine of a free-living rabbit. This lesion was classified as a mild lesion
H&E Bar=250μm

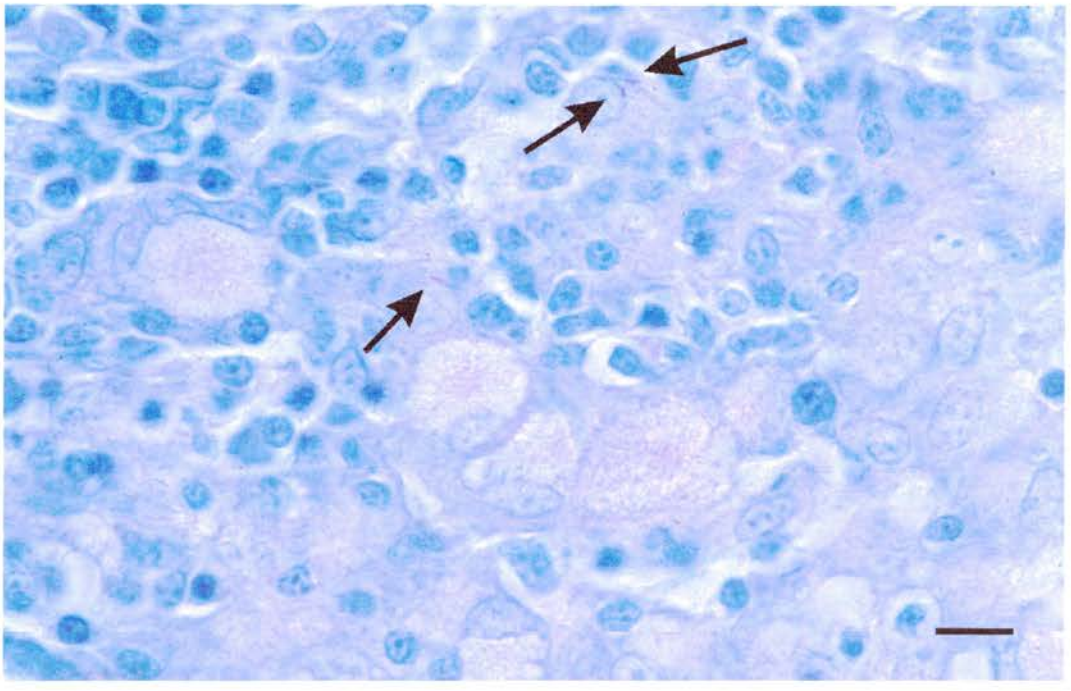


Plate 3,8 Higher magnification of the granuloma in plate 3,7 above, with three AFB visible (arrows)
ZN Bar=100μm

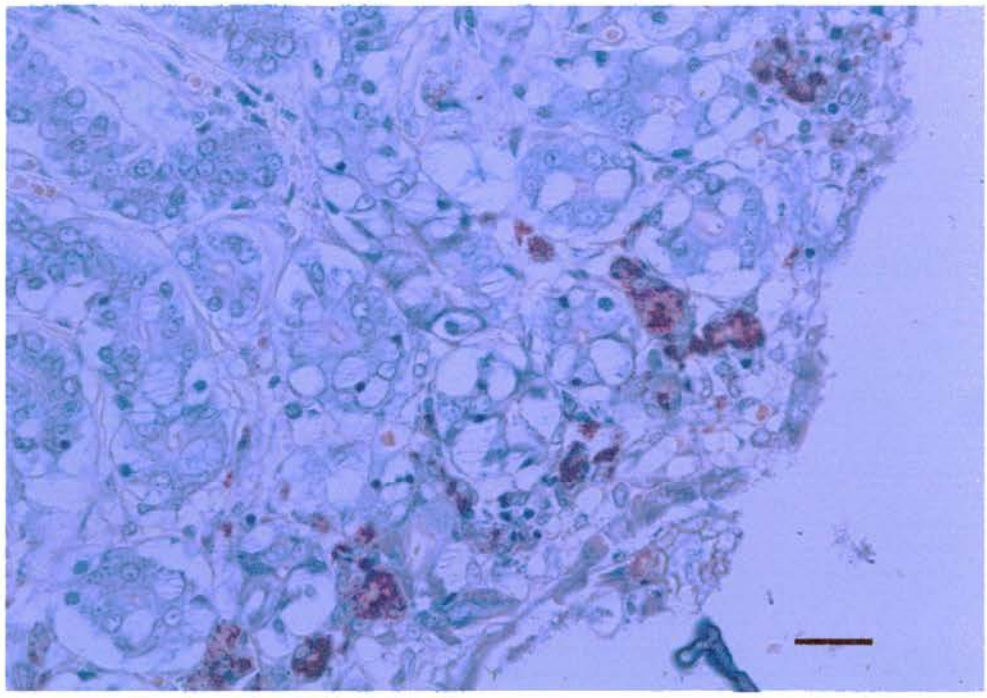


Plate 3,9 A section of colon from a severely affected rabbit, showing AFB in the superficial lamina propria
ZN Bar=25μm

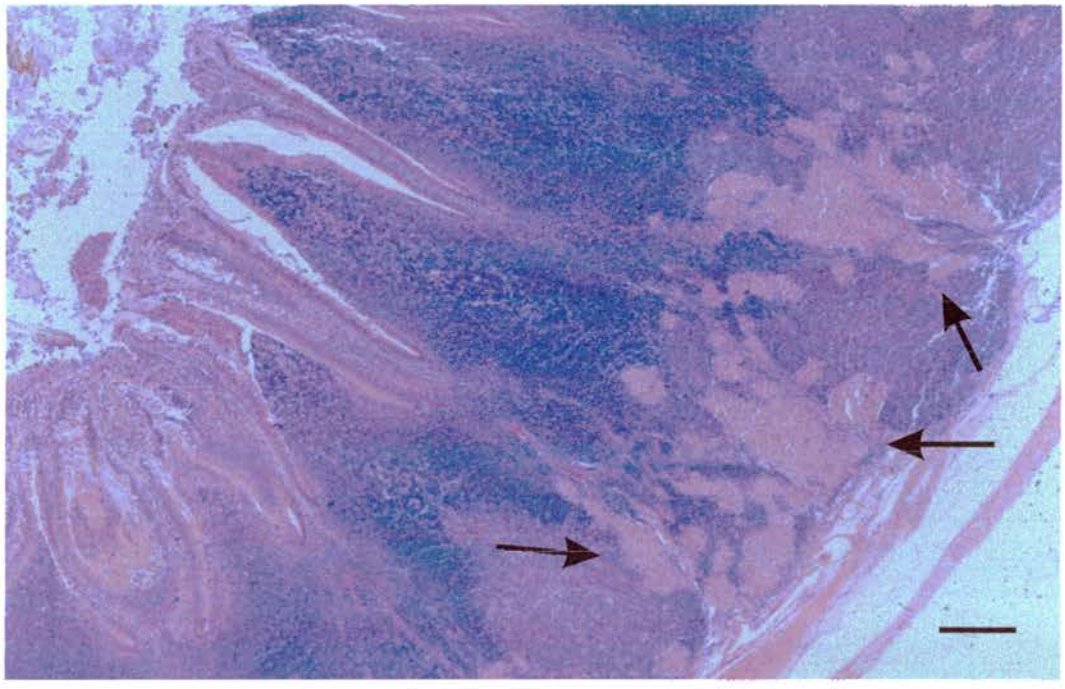


Plate 3,10 A section of the appendix of a severely affected rabbit, showing the distribution of granulomata (arrows) at the base of the lymphoid follicles
H&E Bar=250μm

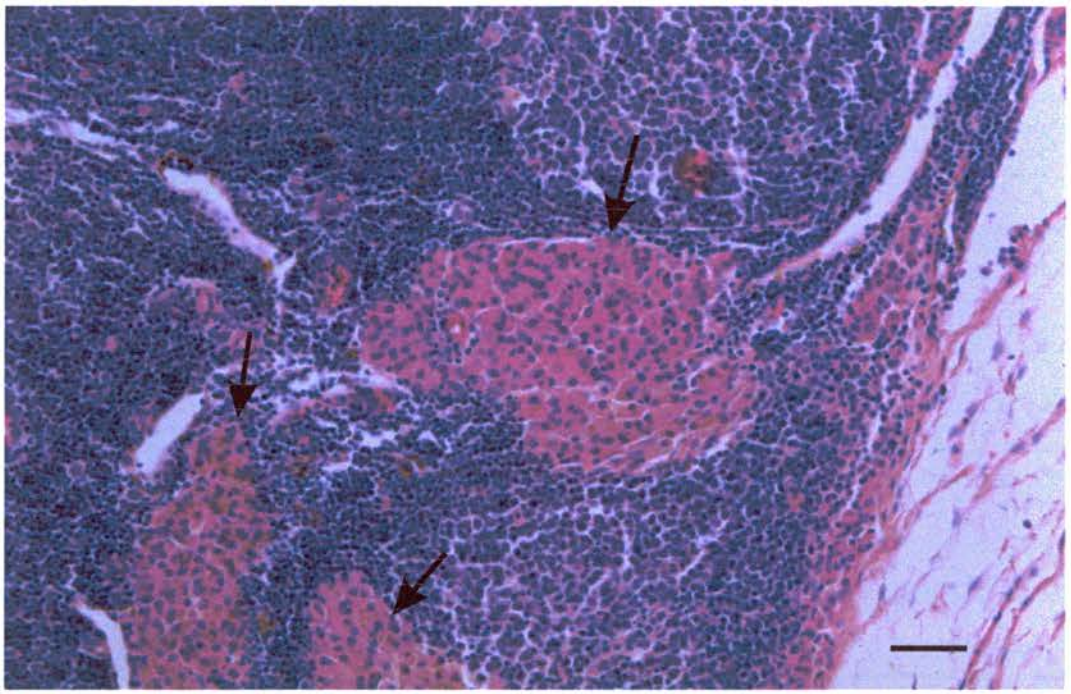


Plate 3,11 The base of a lymphoid follicle in the appendix of a severely affected rabbit, containing numerous macrophages arranged in discrete foci (arrows).
H&E Bar=50μm

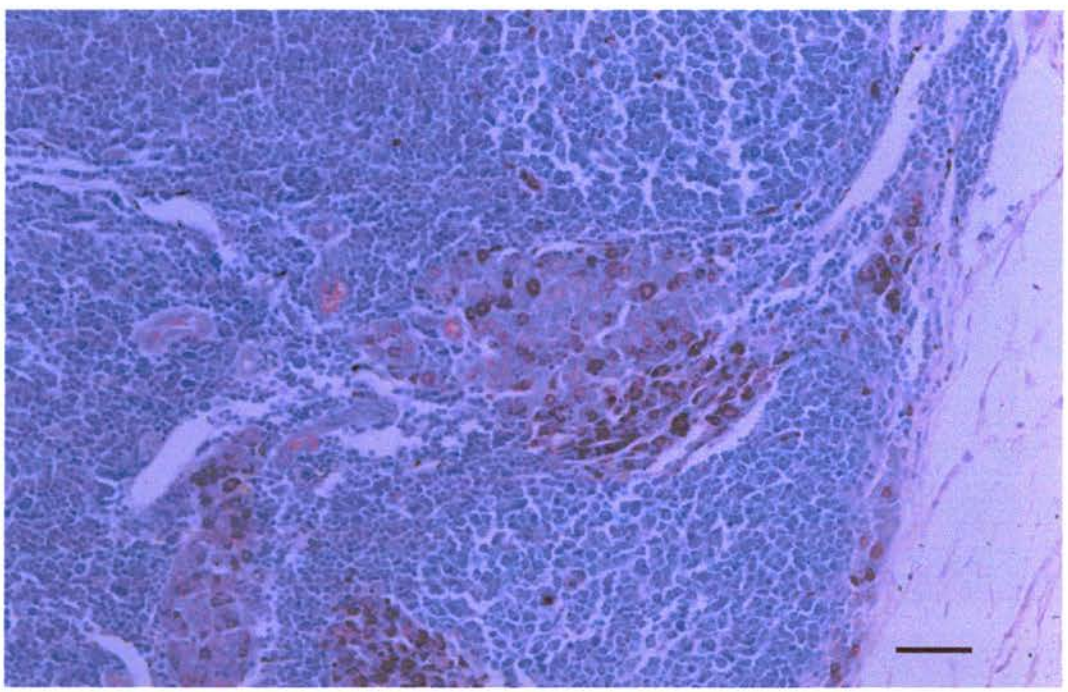


Plate 3,12 A serial section of plate 3,11, showing AFB in the macrophages in the appendix of a severely affected rabbit.
ZN Bar=50μm

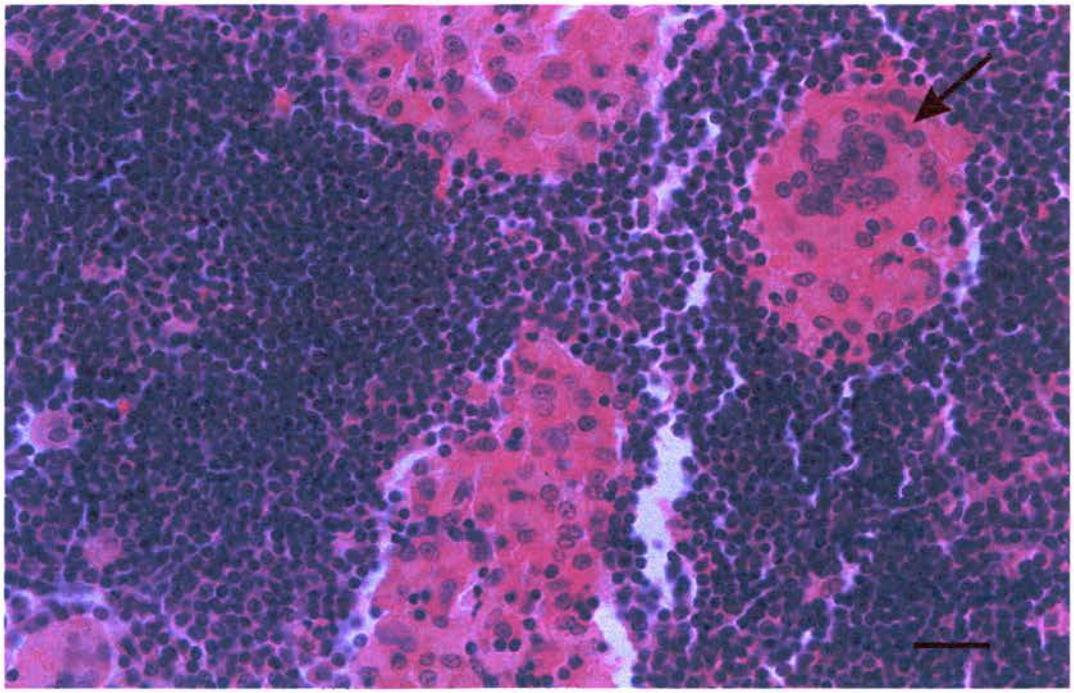
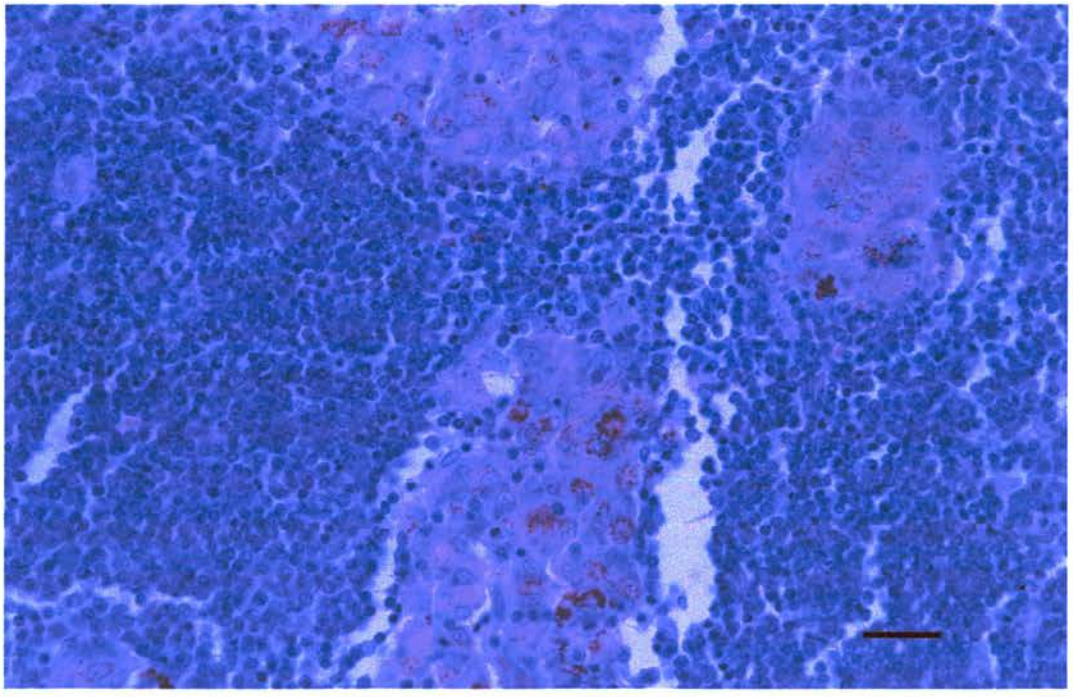


Plate 3,13 A section from the cortex of a MLN from a severely affected rabbit, showing granulomata composed mainly of macrophages, with a giant cell visible (arrow)
H&E Bar=25μm



3,14 A serial section of plate 3,13 above, showing AFB present in the cells of the granulomata
ZN Bar=25μm

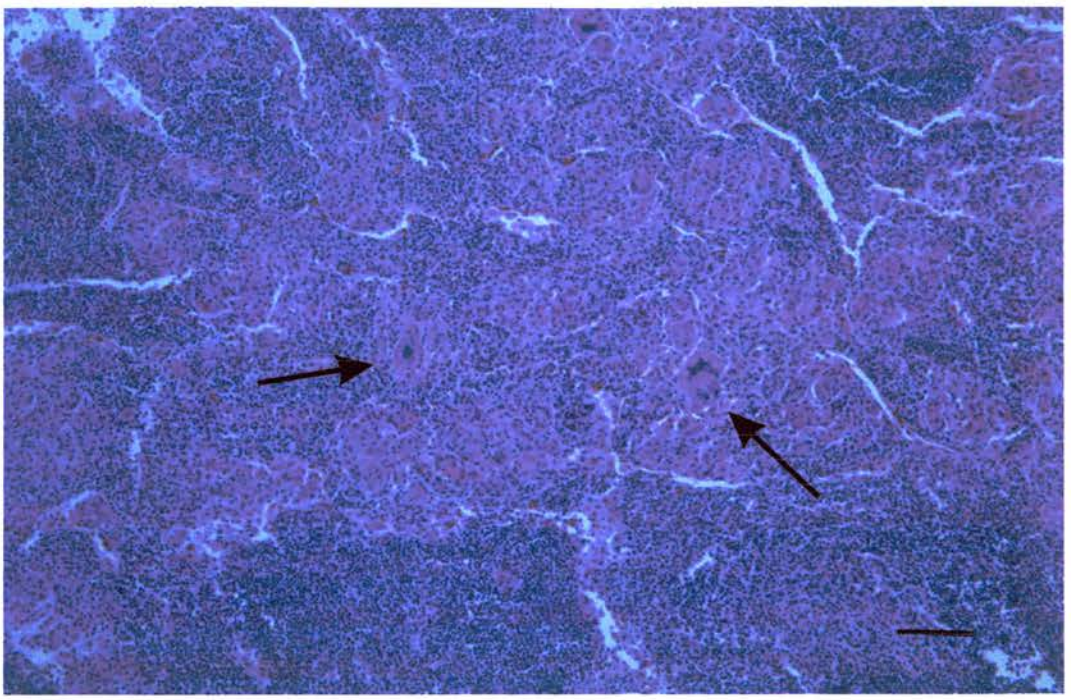


Plate 3,15 A section of the cortex of MLN from rabbit number 22, showing numerous macrophages and giant cells (arrows).
H&E Bar=100μm

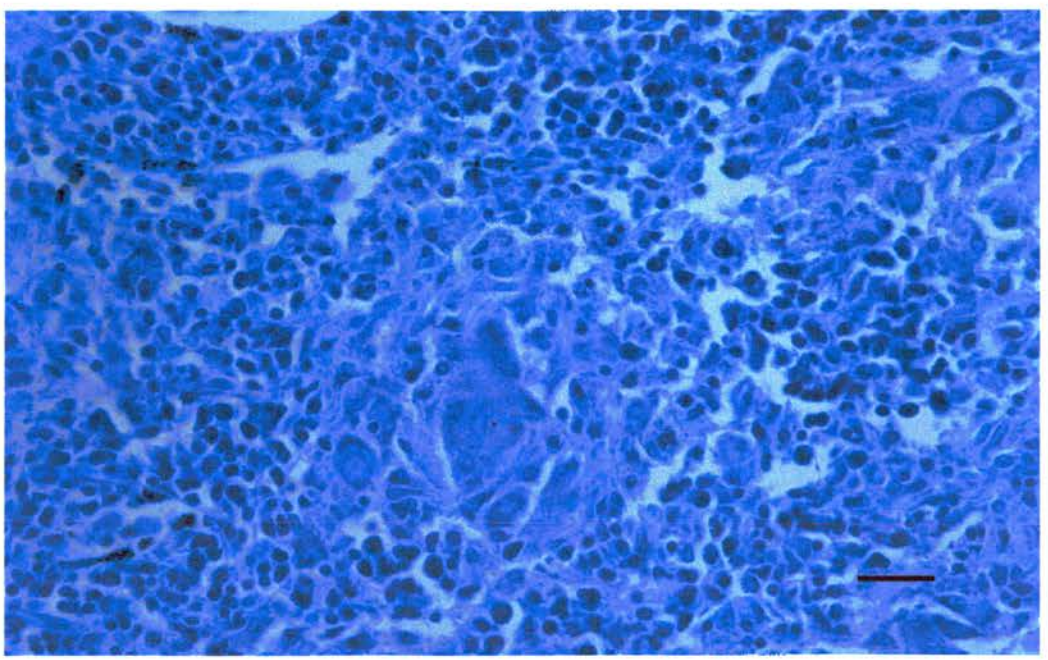


Plate 3,16 Higher magnification of a section of MLN (plate 3,15) from rabbit number 22, with a small number of AFB visible in the chronic inflammatory cells.
ZN Bar=25μm

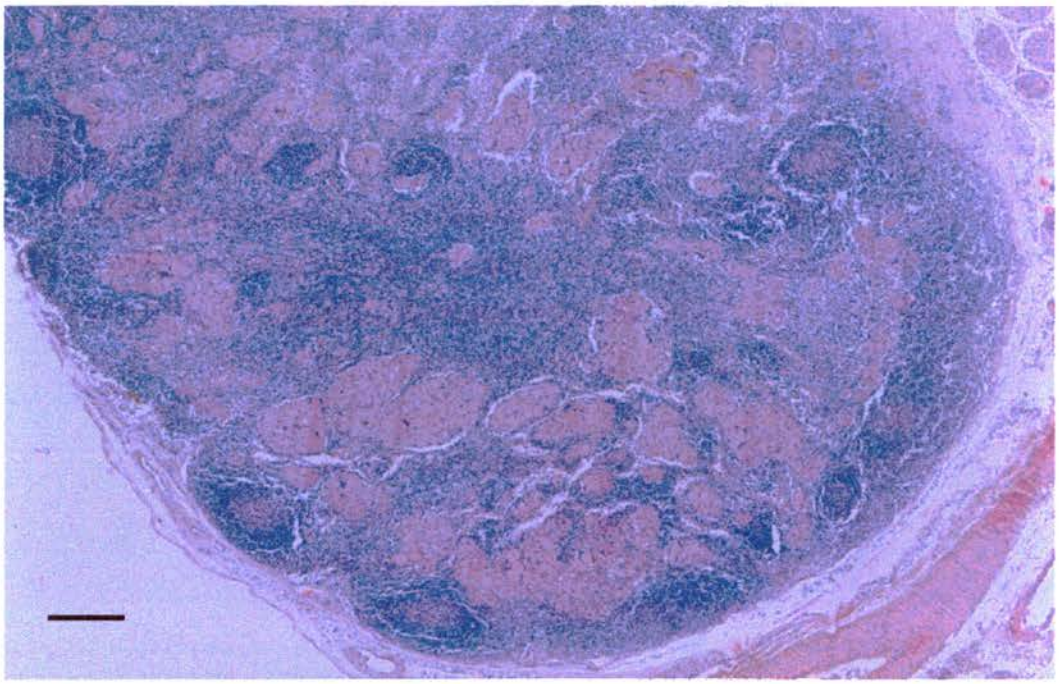


Plate 3,17 A section of MLN from a severely affected rabbit, showing multiple foci of chronic inflammatory cells in the cortex, visible as pale pink staining areas amongst the darker nuclei of the lymphocytes
H&E Bar=250μm

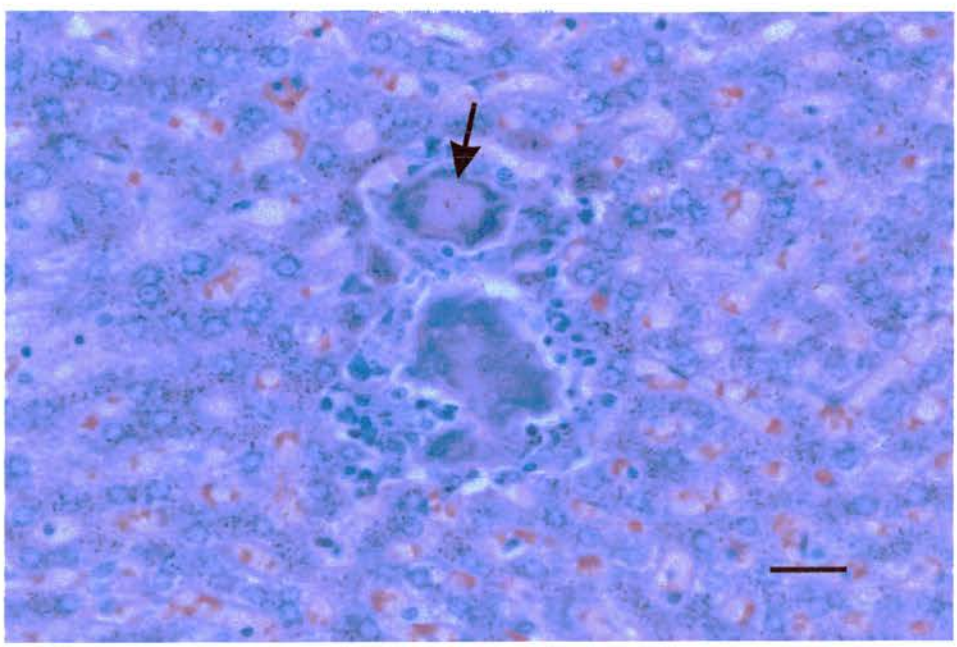


Plate 3,18 A section of liver from a severely affected rabbit, showing a granuloma consisting of two giant cells and a small number of lymphocytes. A few intracellular AFB are present in the cytoplasm of the giant cells (arrow)
ZN Bar=25μm

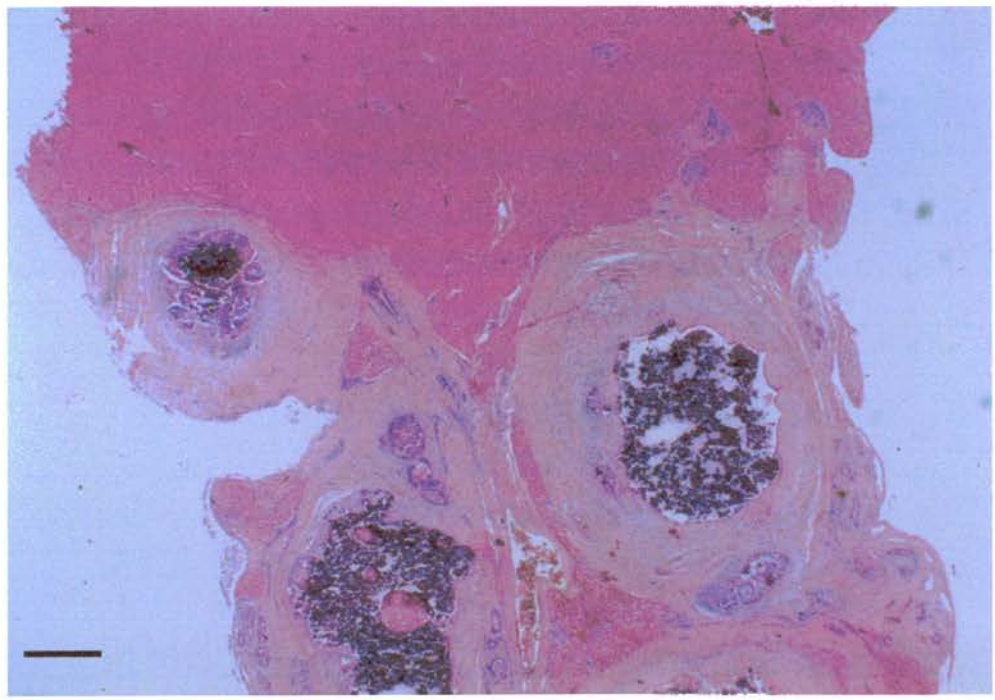


Plate 3,19 A section of liver from a free-living rabbit, showing evidence of hepatic coccidosis. Coccidial oocysts are aggregated within thick walled capsules.
H&E Bar=500μm

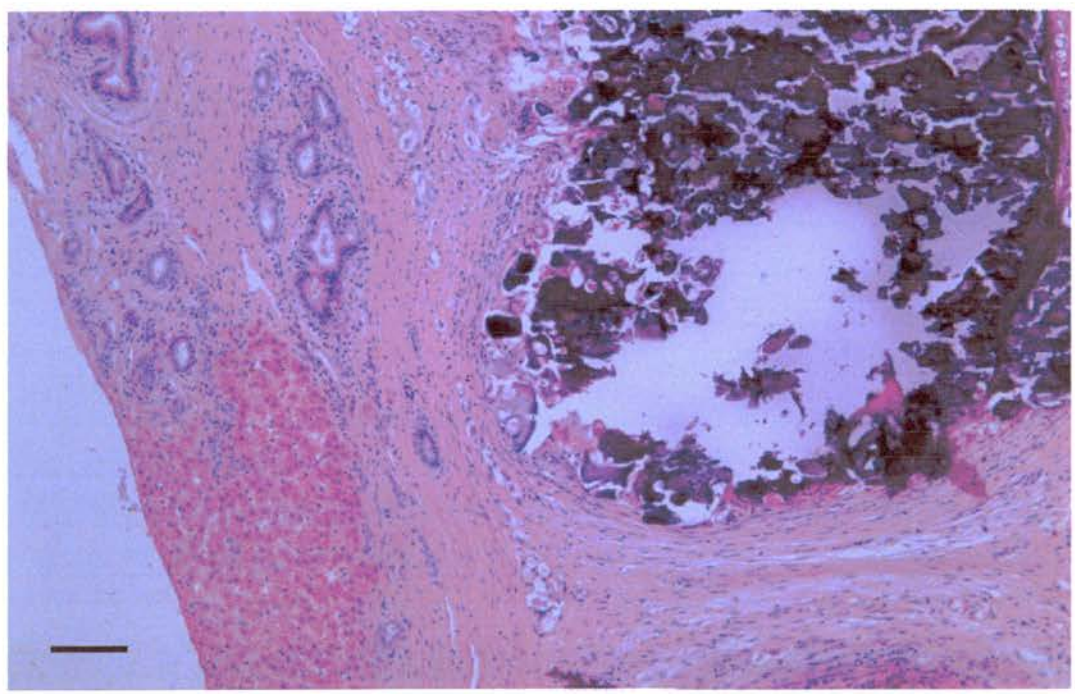


Plate 3,20 Higher magnification of hepatic coccidiosis in a rabbit, revealing extensive bile duct proliferation associated with the fibrous capsule containing mineralised debris.
H&E Bar=100μm

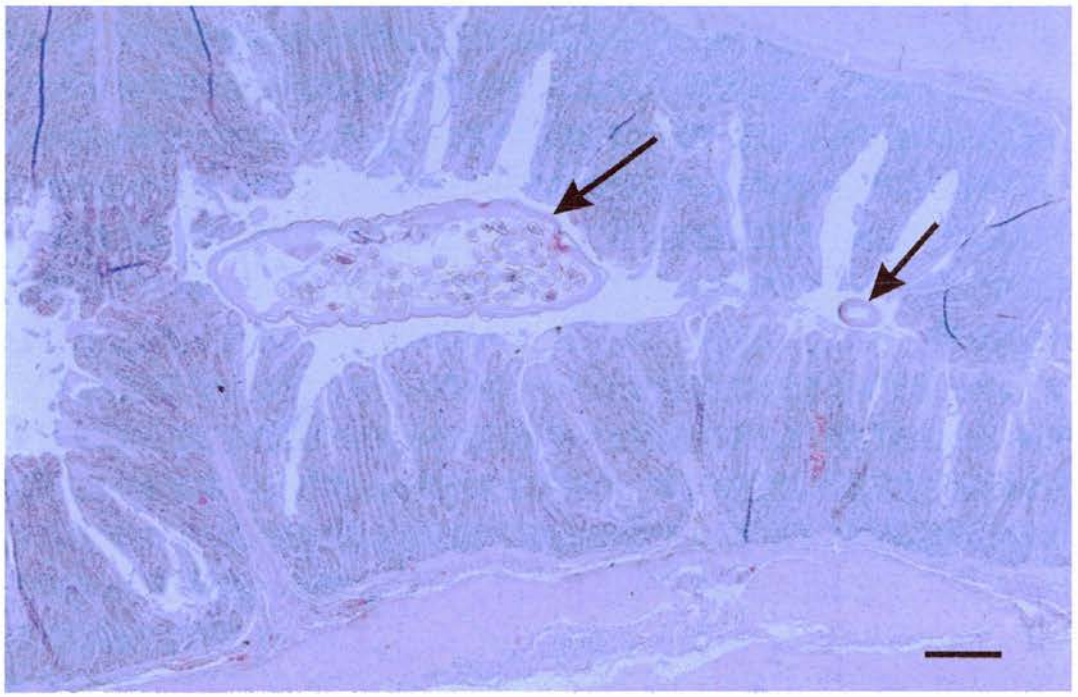


Plate 3,21 An unidentified nematode worm/s (arrows) in the lumen of the intestine of a free-living rabbit
H&E Bar=250μm

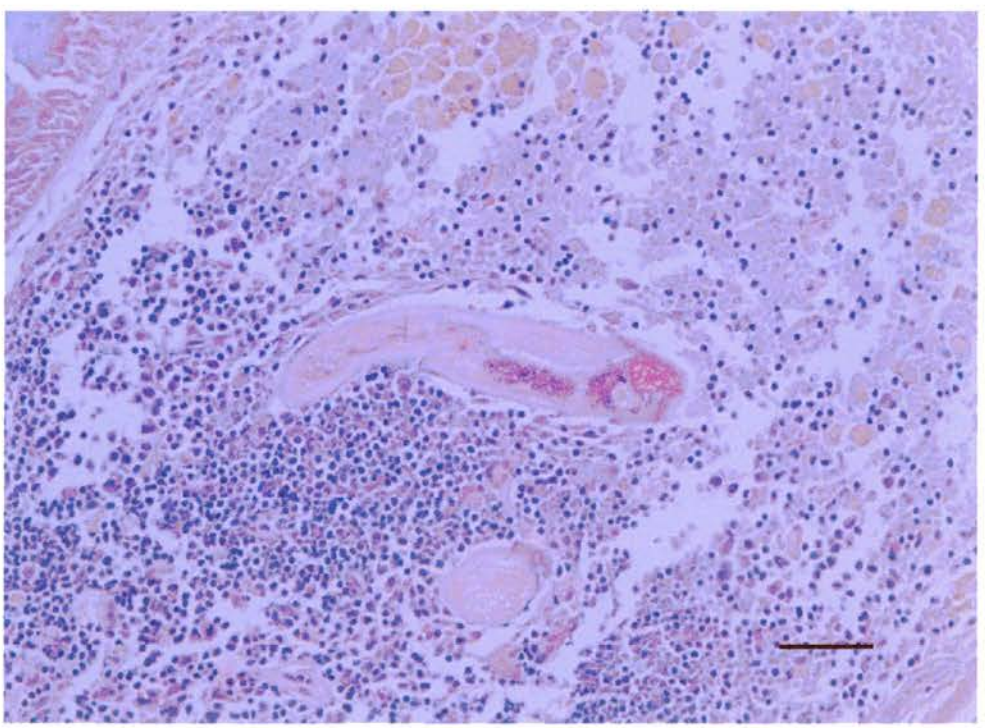


Plate 3,22 An unidentified nematode worm in the GALT of a free-living rabbit
H&E Bar=50μm

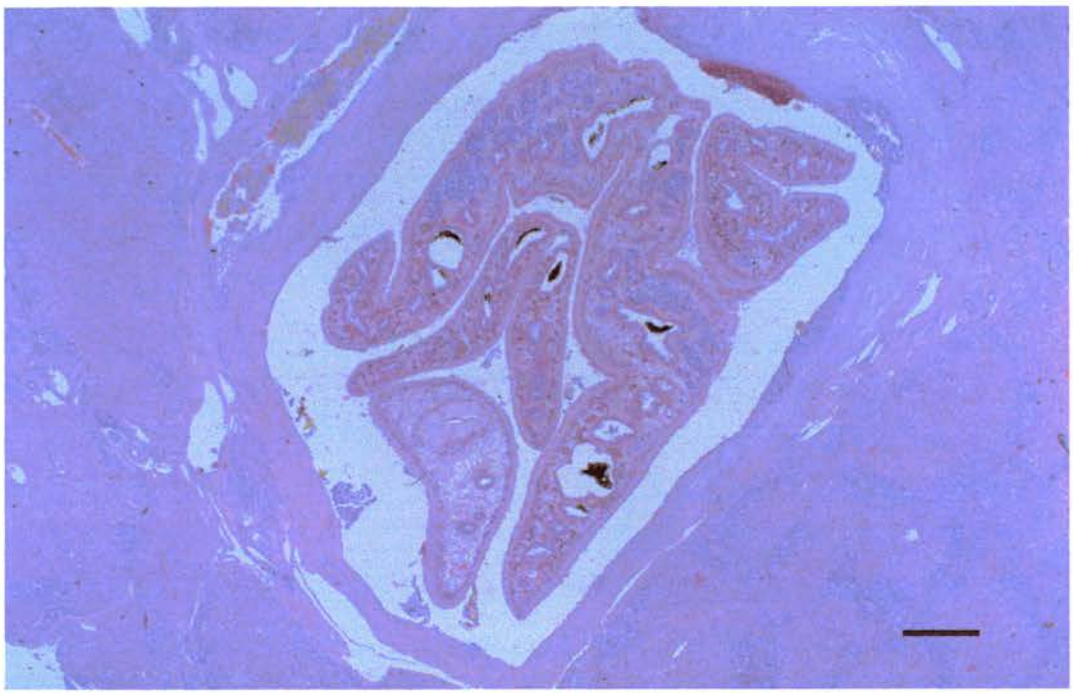


Plate 3,28 A cross section of a liver fluke in the distended bile duct in the liver of a free-living rabbit. The wall of the bile duct is thickened, and areas of inflammation can been seen extending through the parenchyma of the liver H&E Bar=500μm

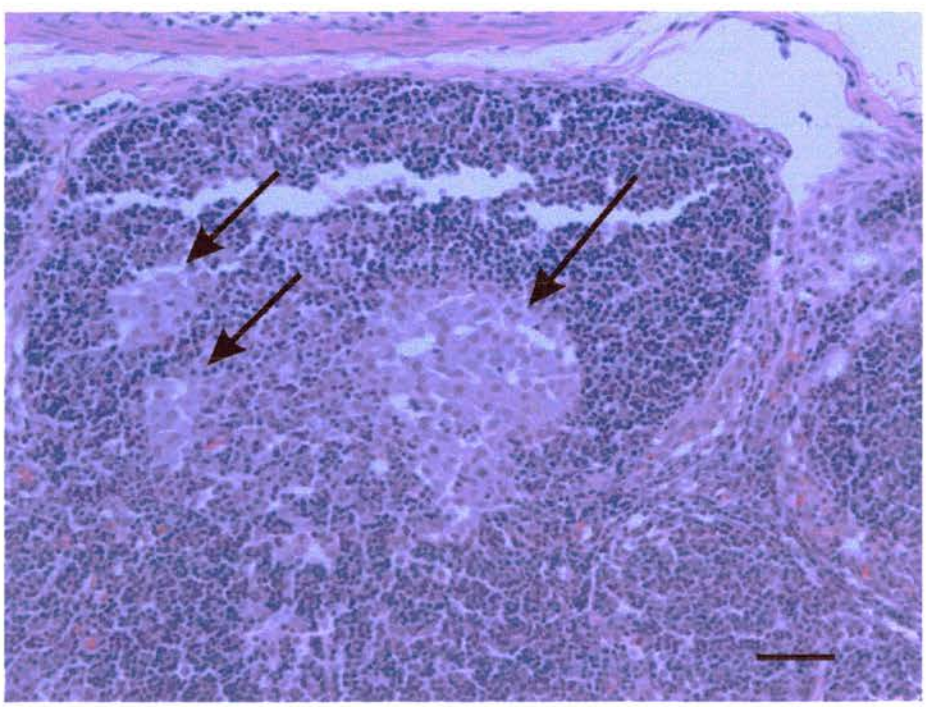


Plate 3,29 A section of lymphoid tissue in the wall of the appendix of a specific pathogen free rabbit, containing aggregates of macrophages (arrows)

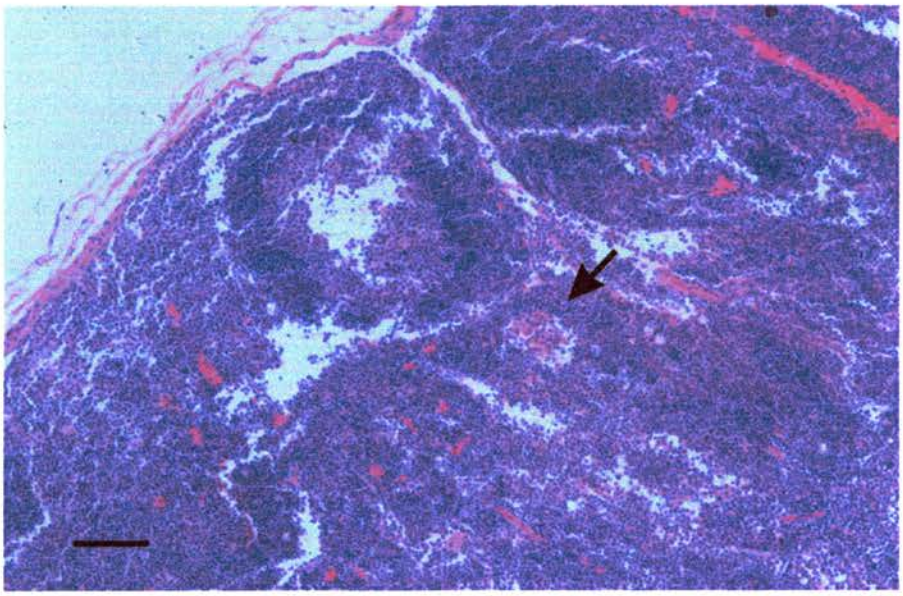


Plate 4,1 A section of the cortex of a MLN from a stoat, with a small granuloma visible (arrow)
H&E Bar=100μm

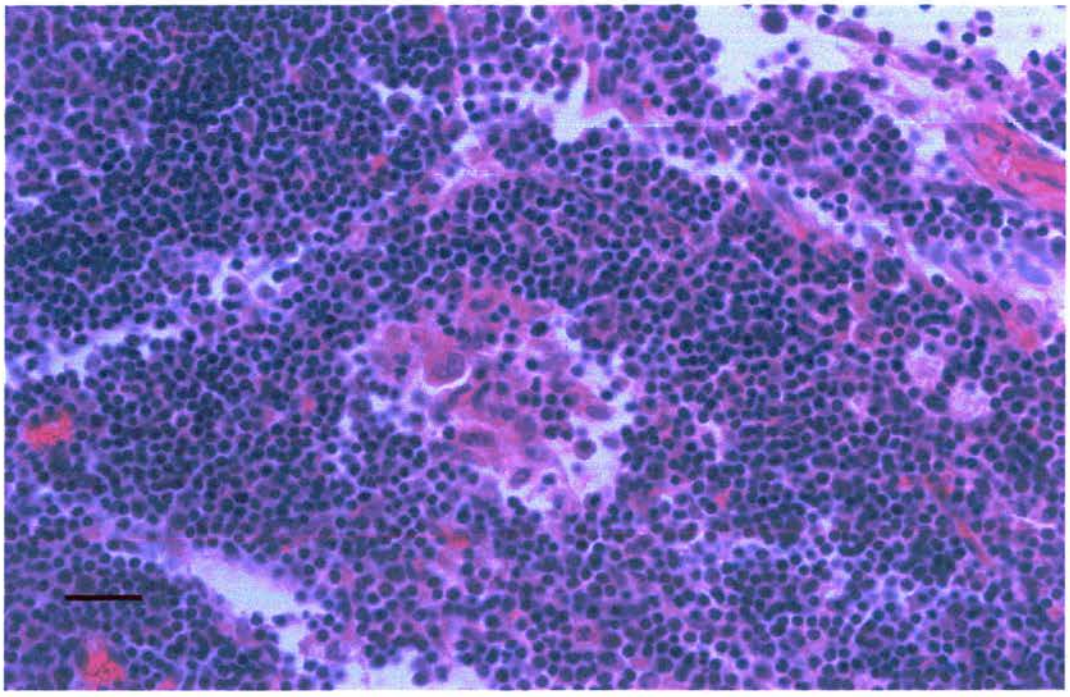


Plate 4,2 Higher magnification of the granuloma in the MLN of a stoat in plate 4,1 above. Note the macrophages with abundant pink foamy cytoplasm.
H&E Bar=25μm

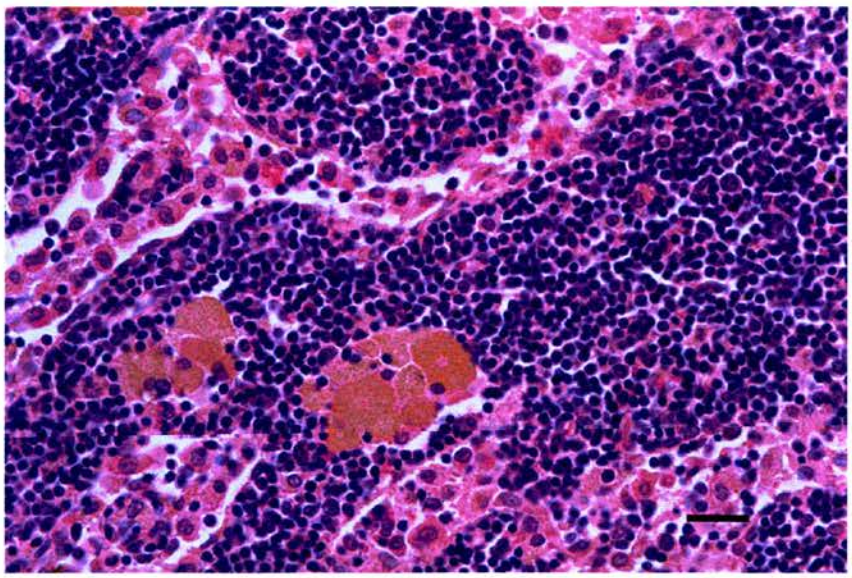
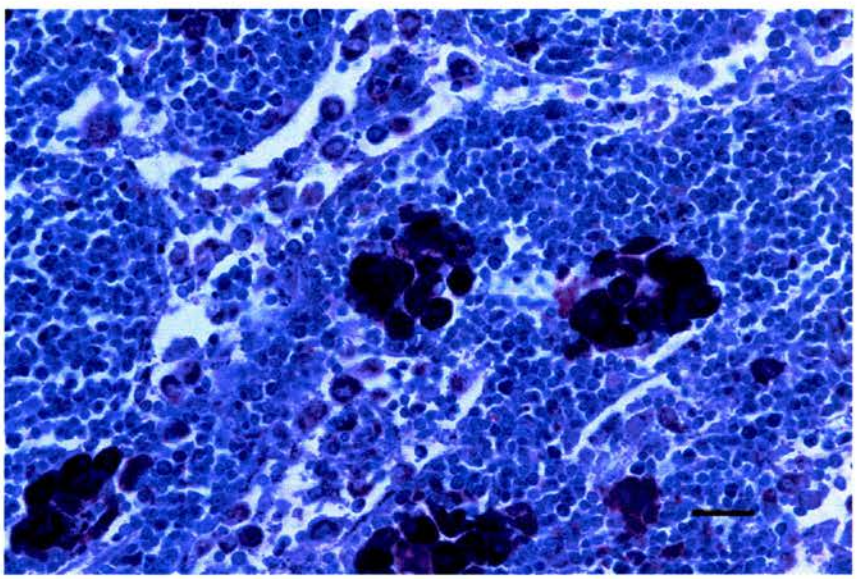


Plate 3,23
Swollen macrophages with yellow intracytoplasmic granules, in the MLN of a free-living rabbit
H&E Bar=25μm

Plate 3,24
Swollen macrophages with dark staining intracytoplasmic granules, in the MLN of a free-living rabbit
ZN Bar=25μm



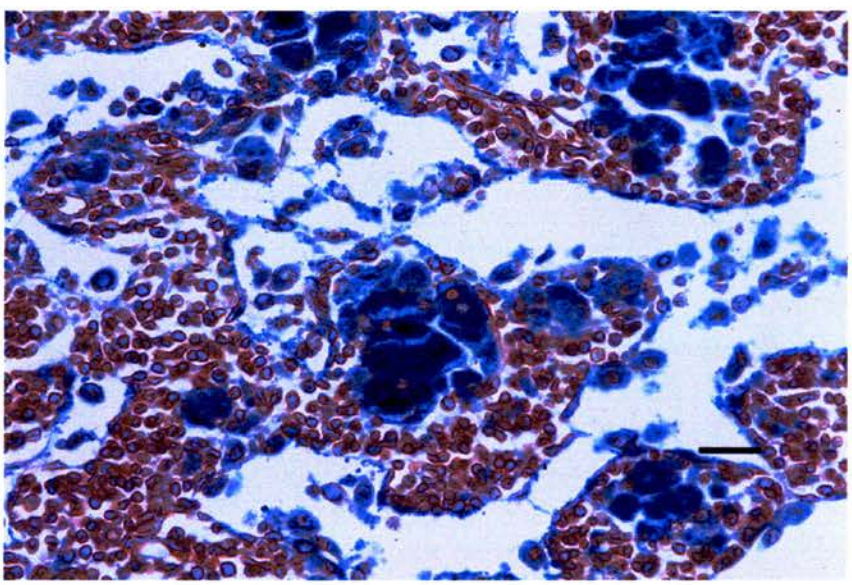


Plate 3,25
Granules in swollen macrophages, staining positively for lipofuscin in a free-living rabbit.
Schmorl's stain
Bar=25μm

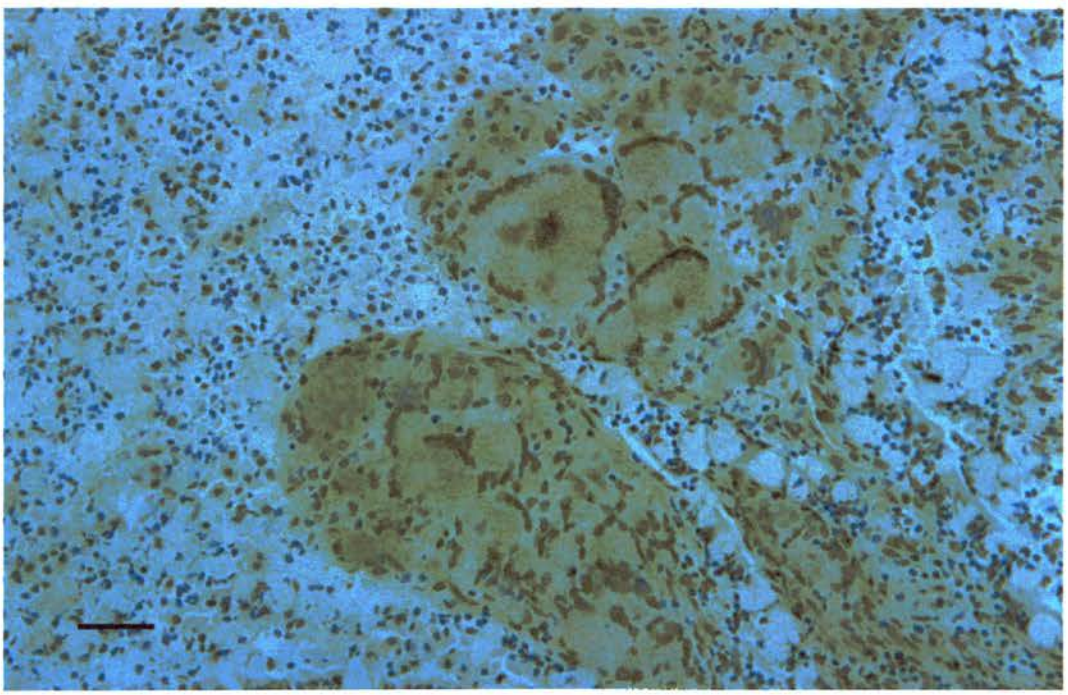


Plate 3,26 A frozen section of the small intestine from a severely affected rabbit, with immunohistochemically labelled *M.a. paratuberculosis* organisms (brown colour) in giant cells and epithelioid cells.
Bar=50μm Method described in chapter 3

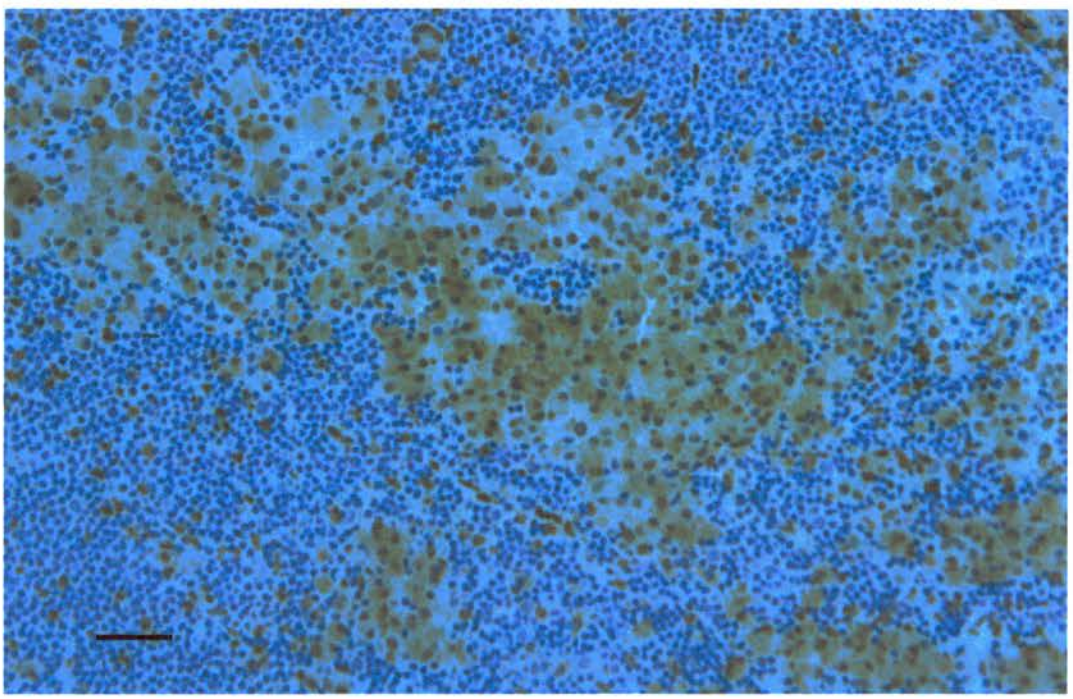


Plate 3,27 Immunohistochemically labelled *M.a. paratuberculosis* organisms in the MLN of a severely affected rabbit.
Bar=50μm Method described in chapter 3

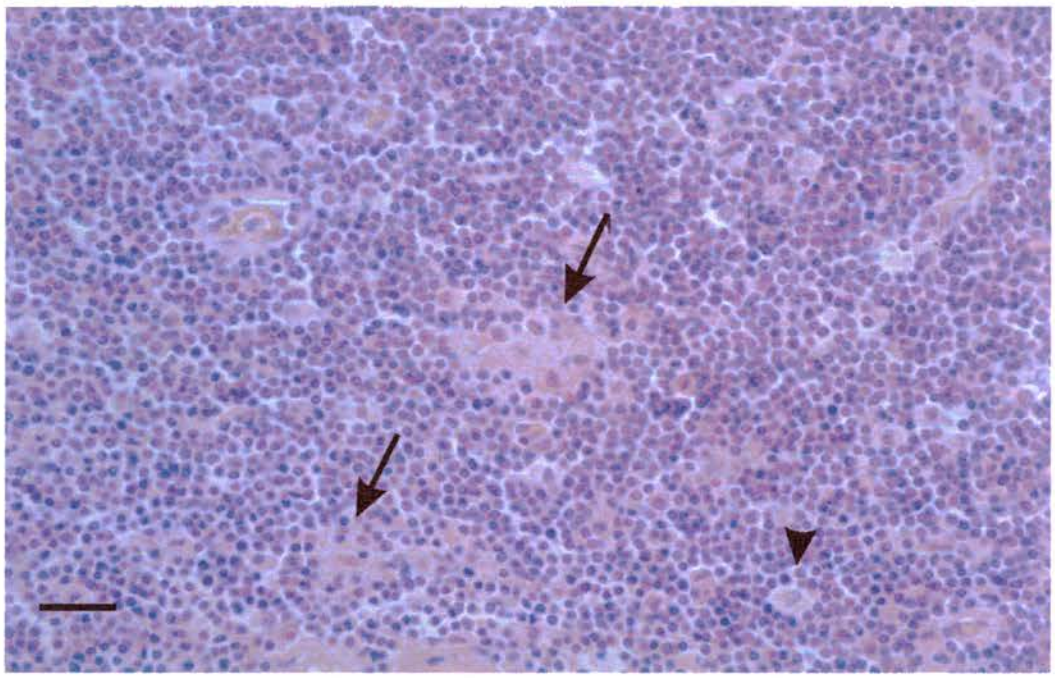


Plate 4,3 A section from the cortex of the MLN of a fox, showing macrophages scattered amongst the lymphocytes (arrowhead) and in small granulomata (arrows)
H&E Bar=50μm

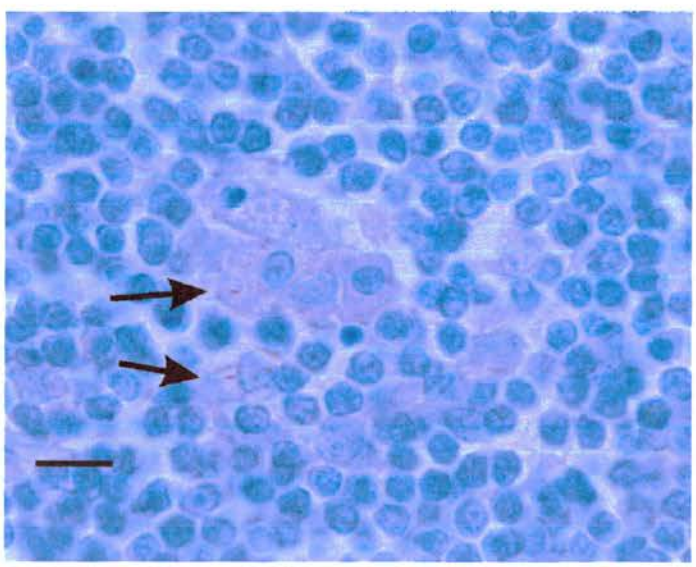


Plate 4,4 A small cluster or macrophages in the MLN of a fox, two intracellular AFB are visible (arrows)
ZN Bar=10μm

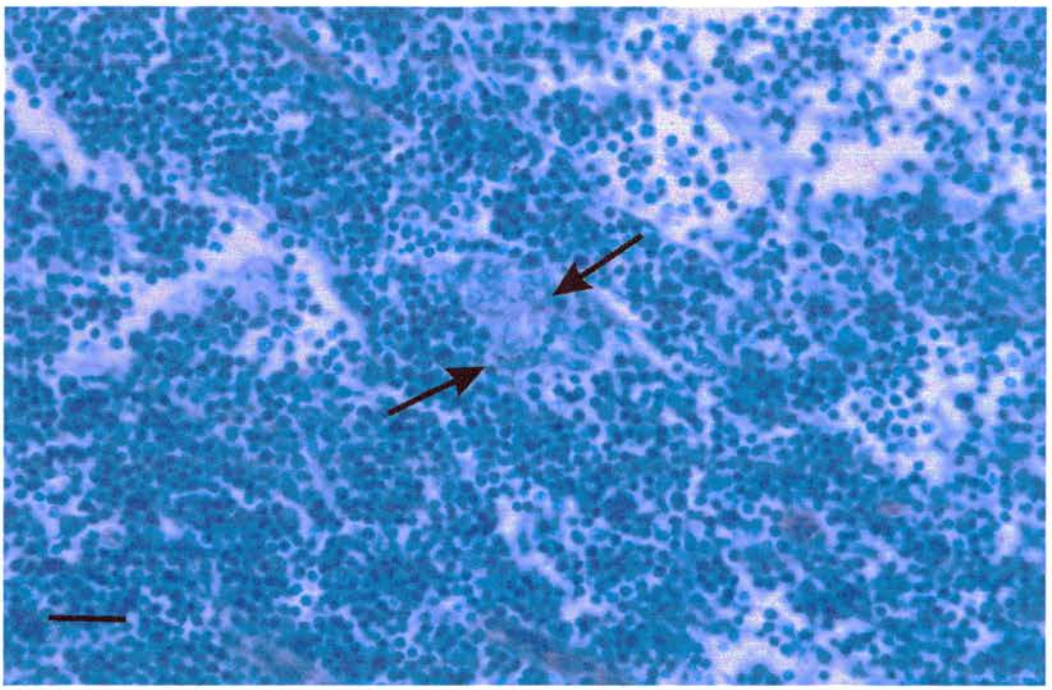


Plate 4,5 A section of MLN from a stoat, showing the granuloma seen in plates 4,1 and 4,2. Two AFB are visible (arrows).
ZN Bar=25μm

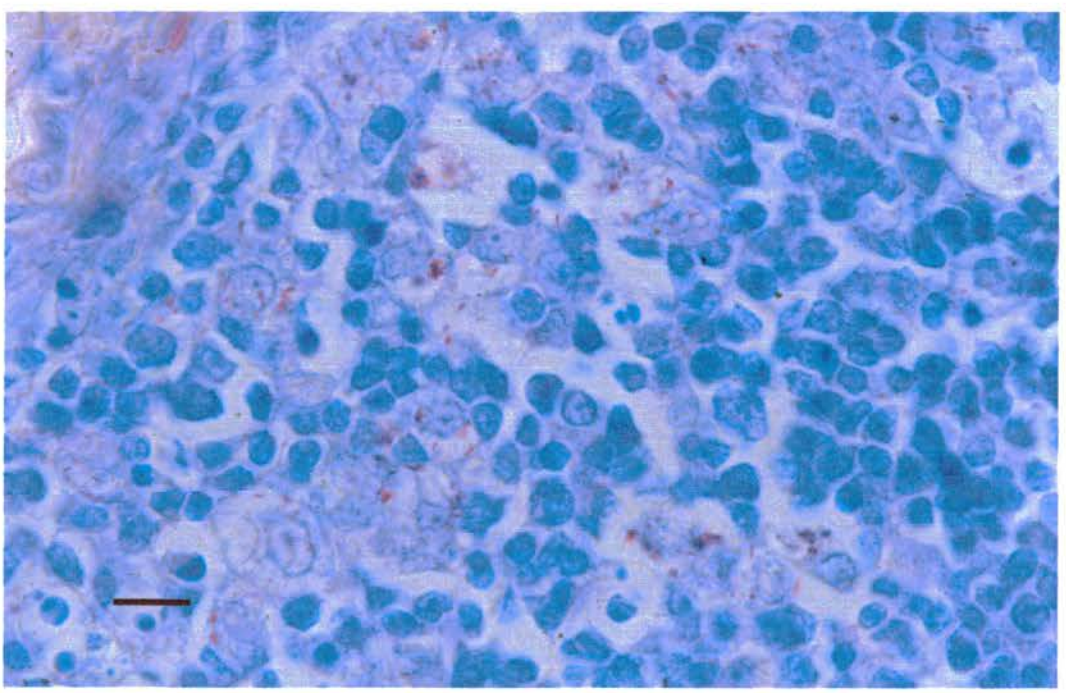


Plate 4,6 Abundant AFB present in the cytoplasm of macrophages in the GALT of a fox.
ZN Bar=10μm

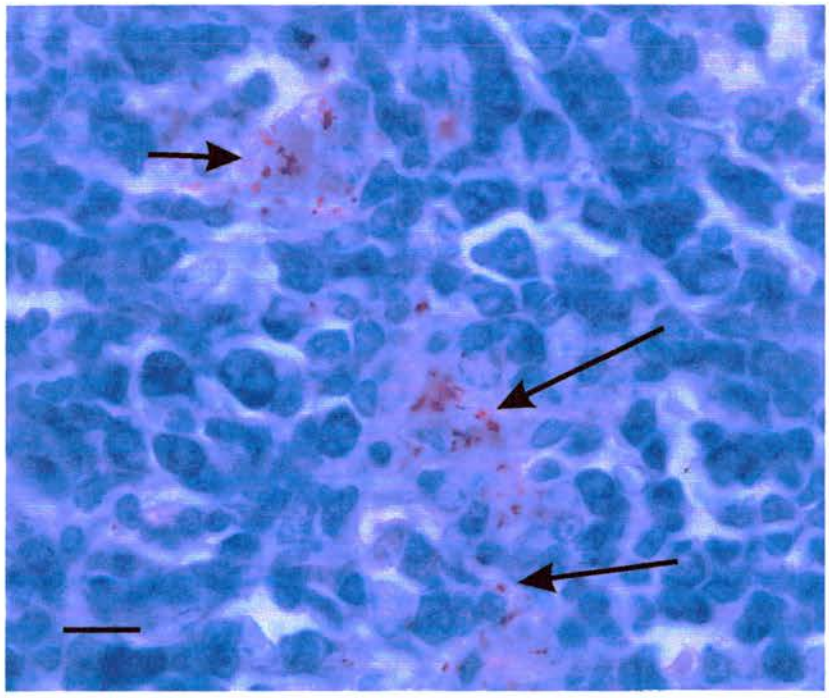


Plate 4,7 A section of cortex of a MLN from a free-living wood mouse, with AFB present (arrows)
ZN Bar=10μm

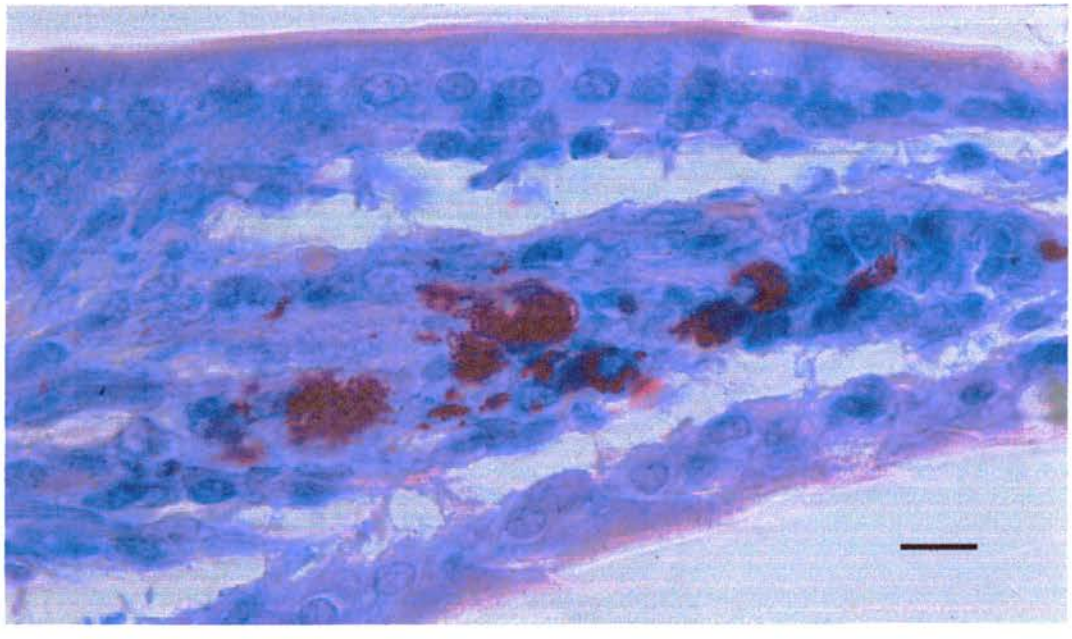


Plate 4,8 Section of a villus from the intestine of a free-living wood mouse. Note the numerous AFB present
ZN Bar=10μm

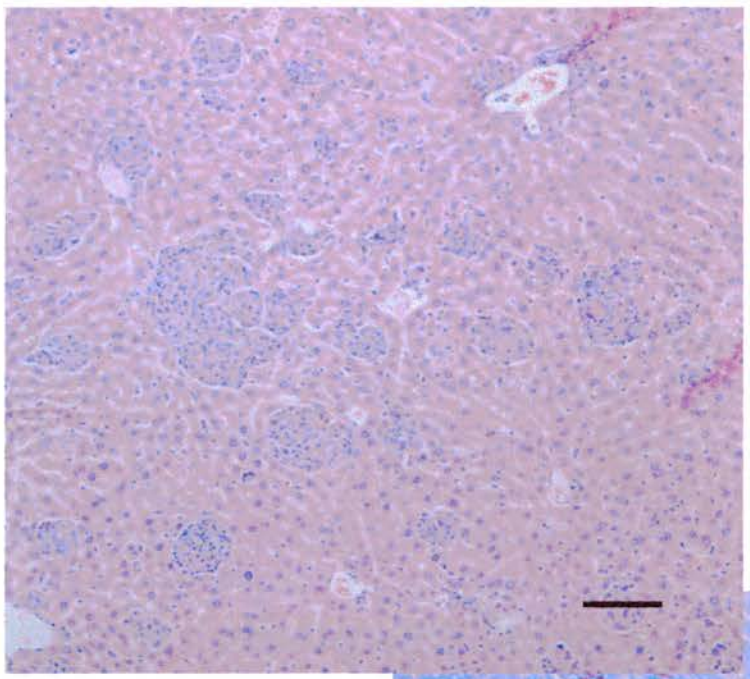


Plate 4,9 A section from the liver of a bank vole, showing numerous granulomata scattered throughout the parenchyma
H&E Bar=100μm

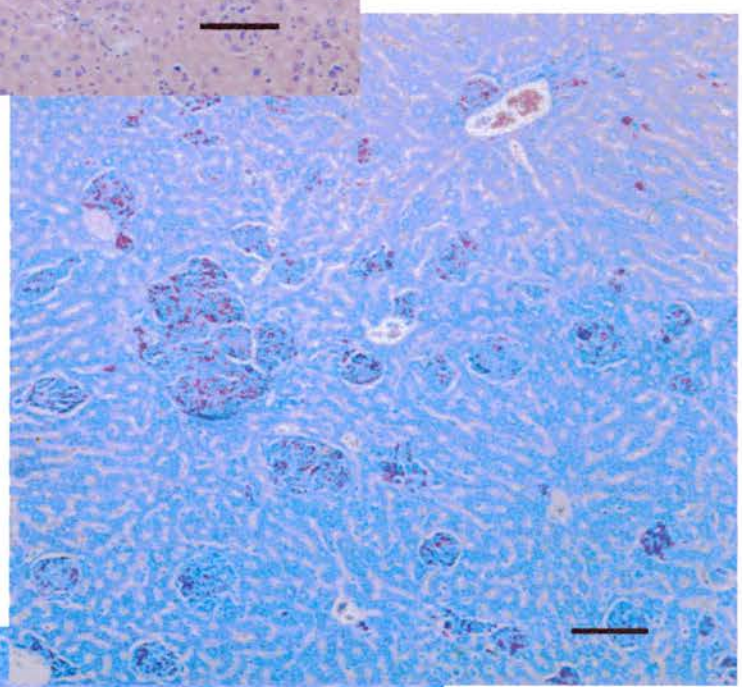


Plate 4,10 A serial section of the sample of liver in plate 4,9 above, showing abundant AFB present in the granulomata
ZN Bar=100μm

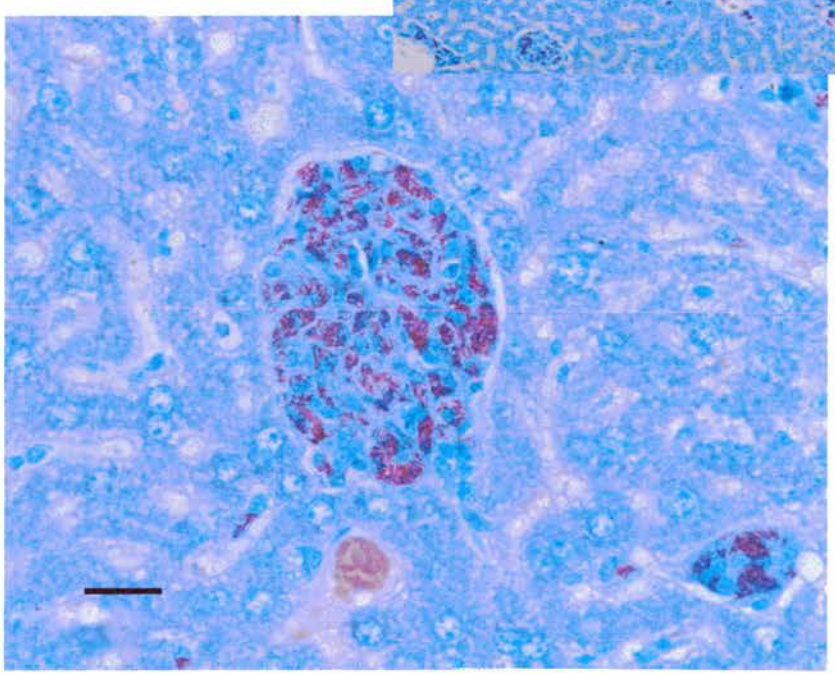


Plate 4,11 Higher magnification of plate 4,10 above, showing a granuloma in the liver of bank vole, from plate 4,10
ZN Bar=25μm

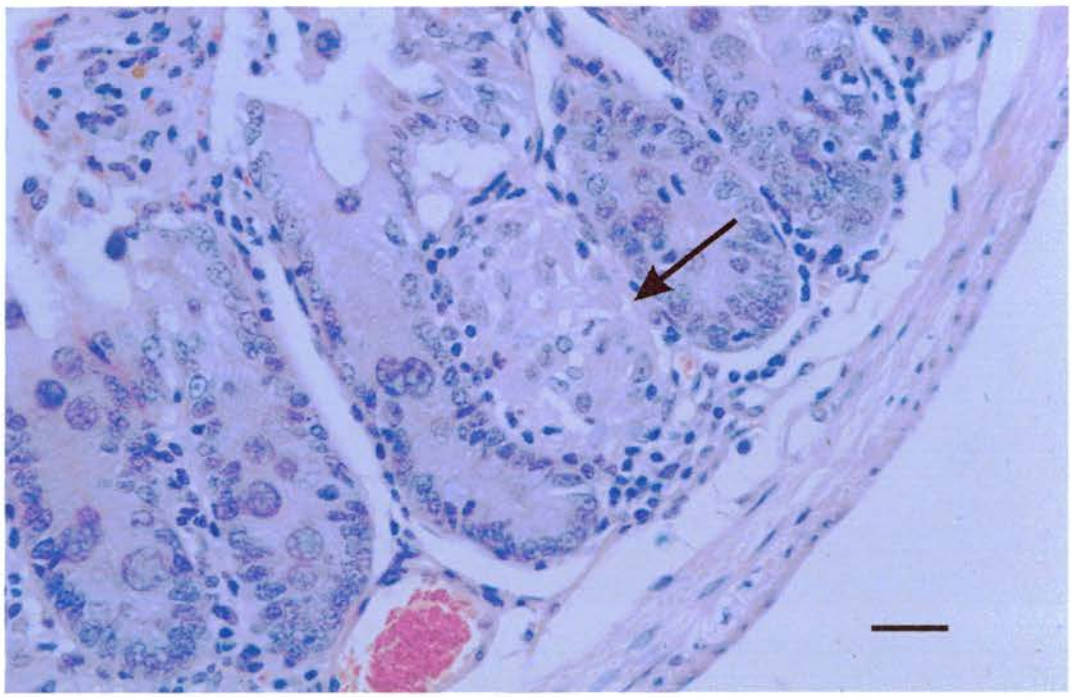


Plate 4,12 The base of the villi in the intestine of a bank vole, with a small granuloma (arrow) visible. The liver of this animal is shown in plates 4,9-4,11
H&E Bar=25μm

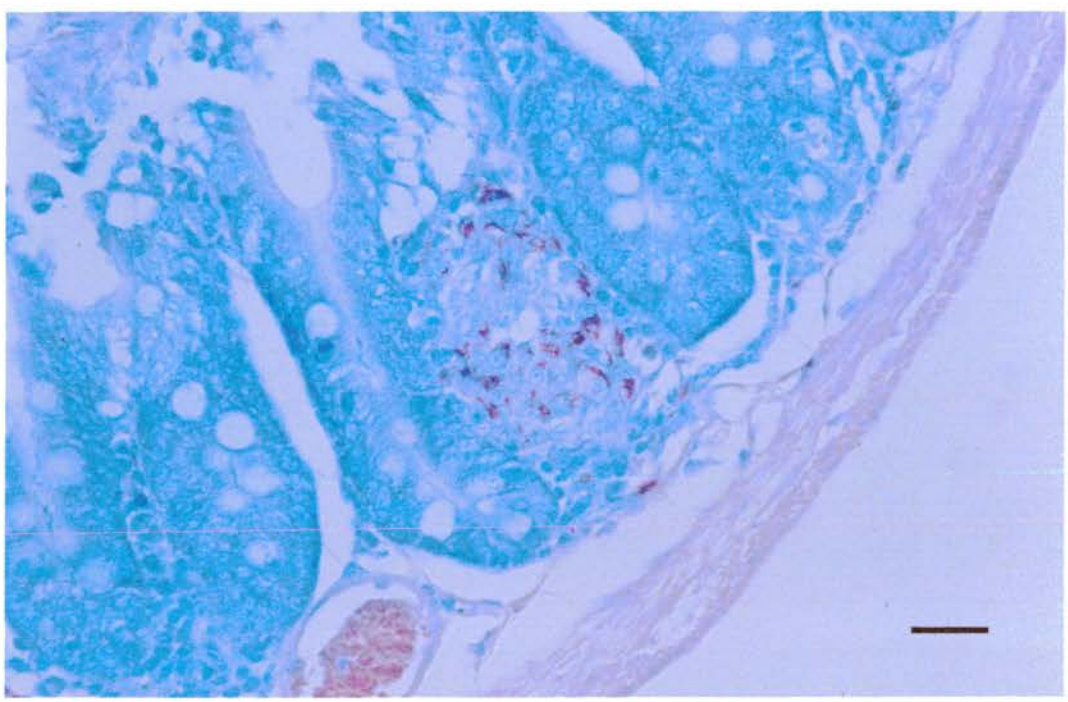


Plate 4,13 A serial section of plate 4,12 above, showing AFB present in the granuloma in the intestine of the bank vole
ZN Bar=25μm

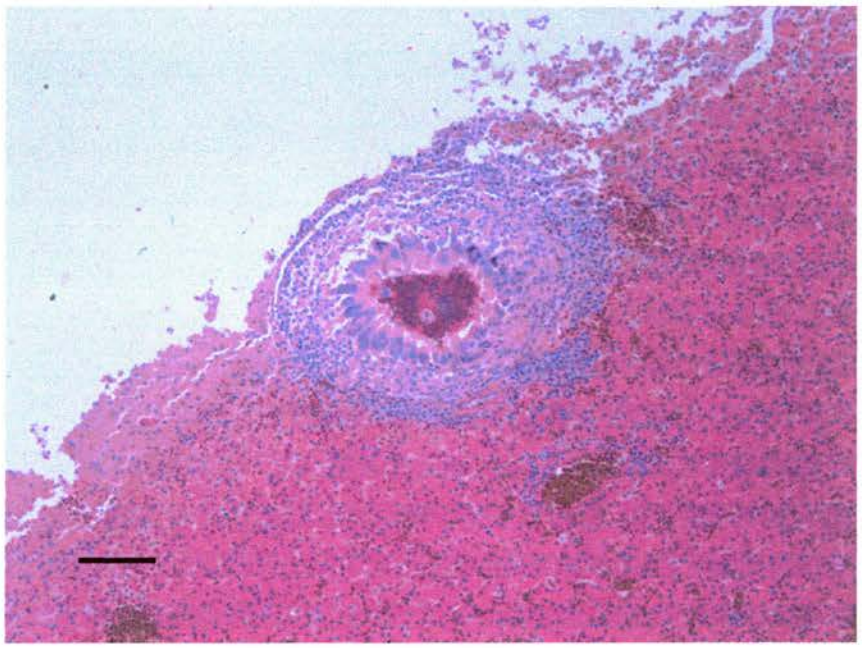


Plate 4,14 A granuloma on the surface of the liver of a buzzard
H&E Bar=100μm

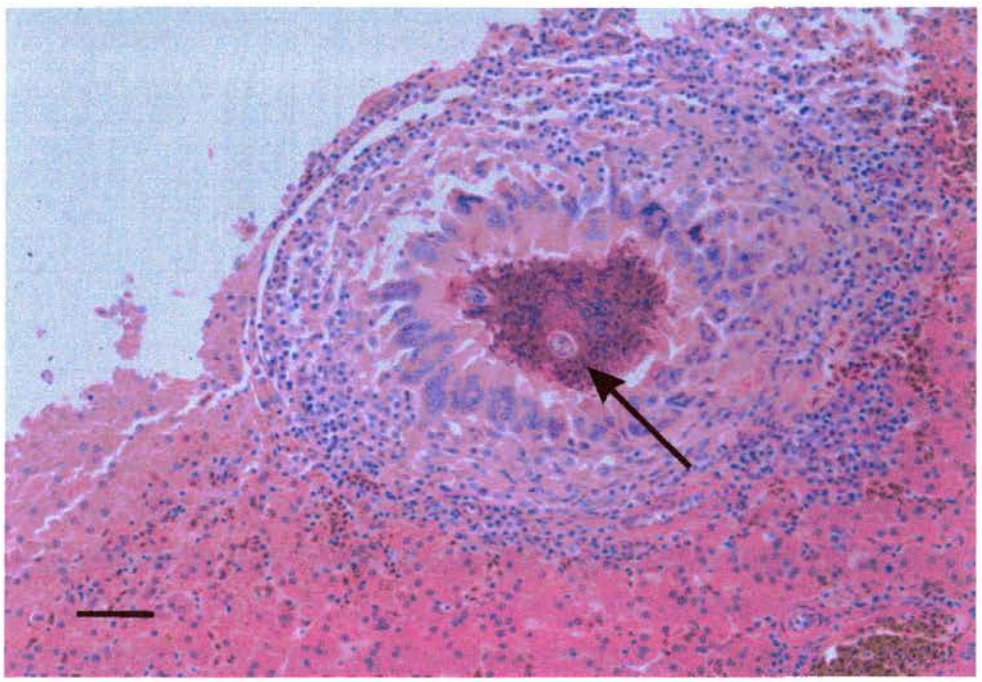


Plate 4,15 Higher powered magnification of the liver from a buzzard (plate 4,14), showing a granulomatous reaction around a foreign body, possibly a parasite (arrow).
H&E Bar=50μm

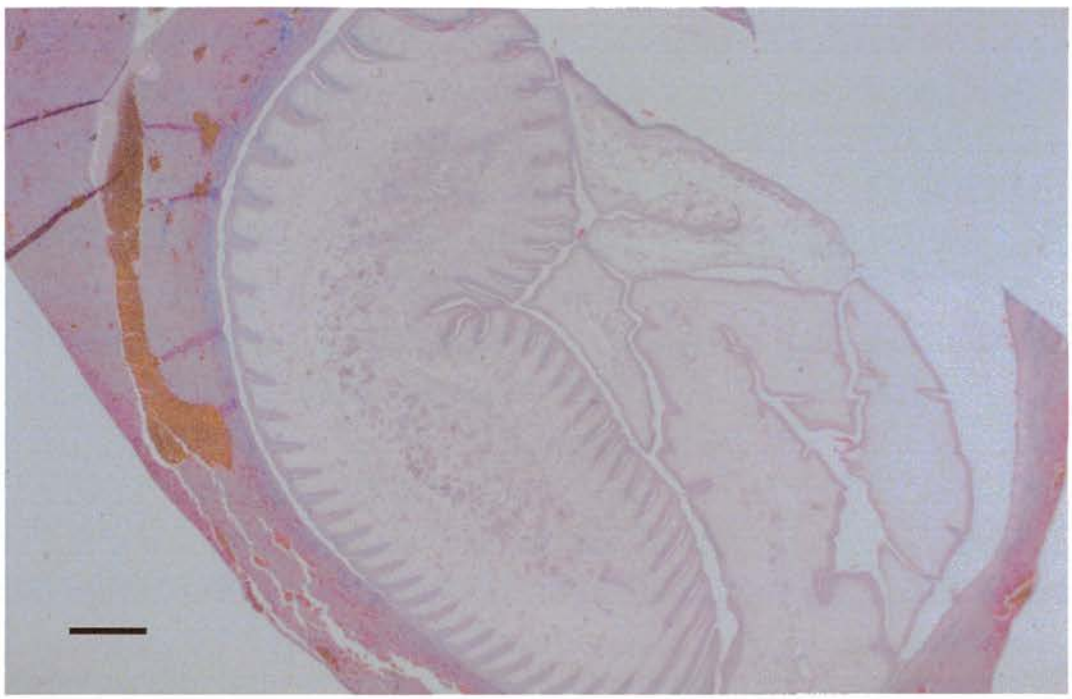


Plate 4,16 A section of liver from a field vole, containing a large unidentified parasite, leaving only a rim of normal parenchyma.
H&E Bar=500μm

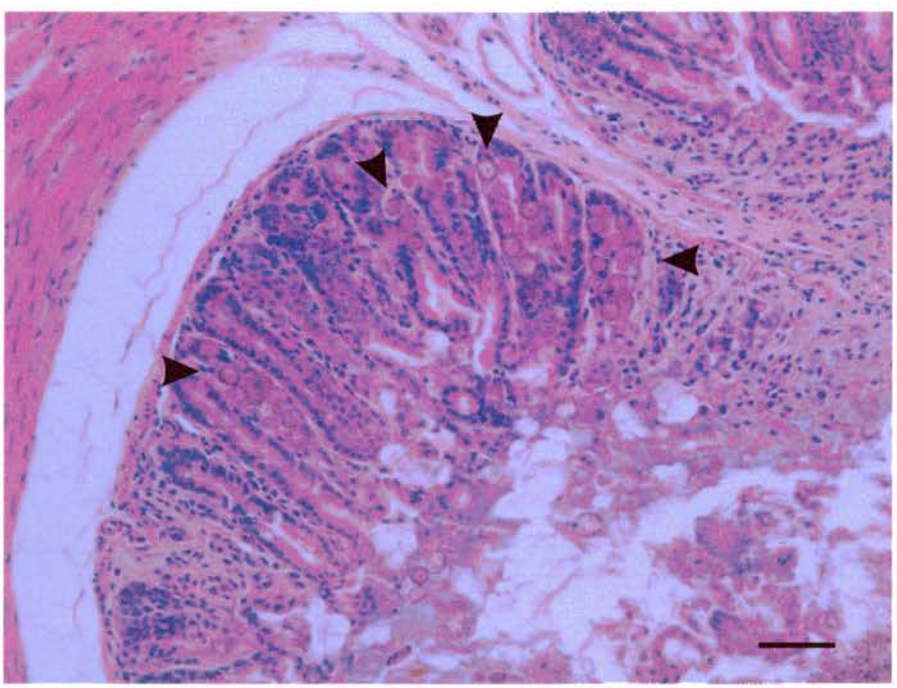


Plate 4,17 A section of the small intestine of a wood mouse, with numerous coccidial oocysts present amongst the epithelial cells (arrowheads)
H&E Bar=50μm

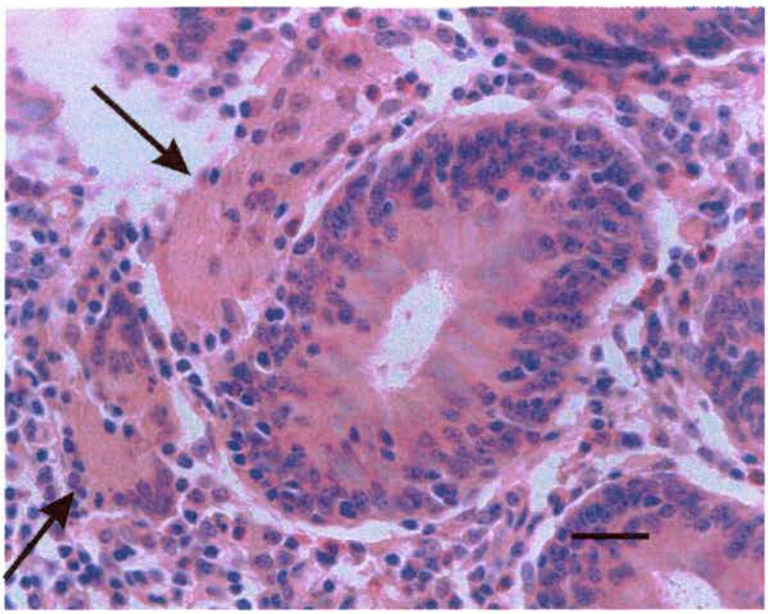


Plate 5,1 A section from the ICV of calf 1522, infected with a leporine isolate of *M.a. paratuberculosis*. Note the chronic inflammatory cells present (arrows) amongst the crypts of the villi.
H&E Bar=25μm

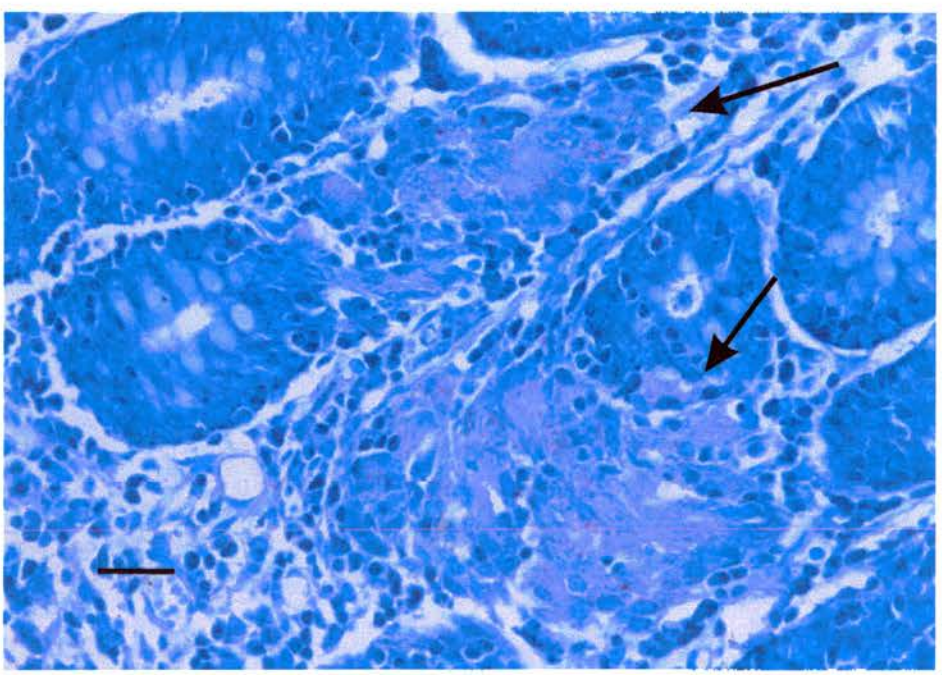


Plate 5,2 A section from the ICV of calf 1522, experimentally infected with a leporine isolate of *M.a. paratuberculosis*. Note the AFB present in chronic inflammatory cells (arrows).
ZN Bar=25μm

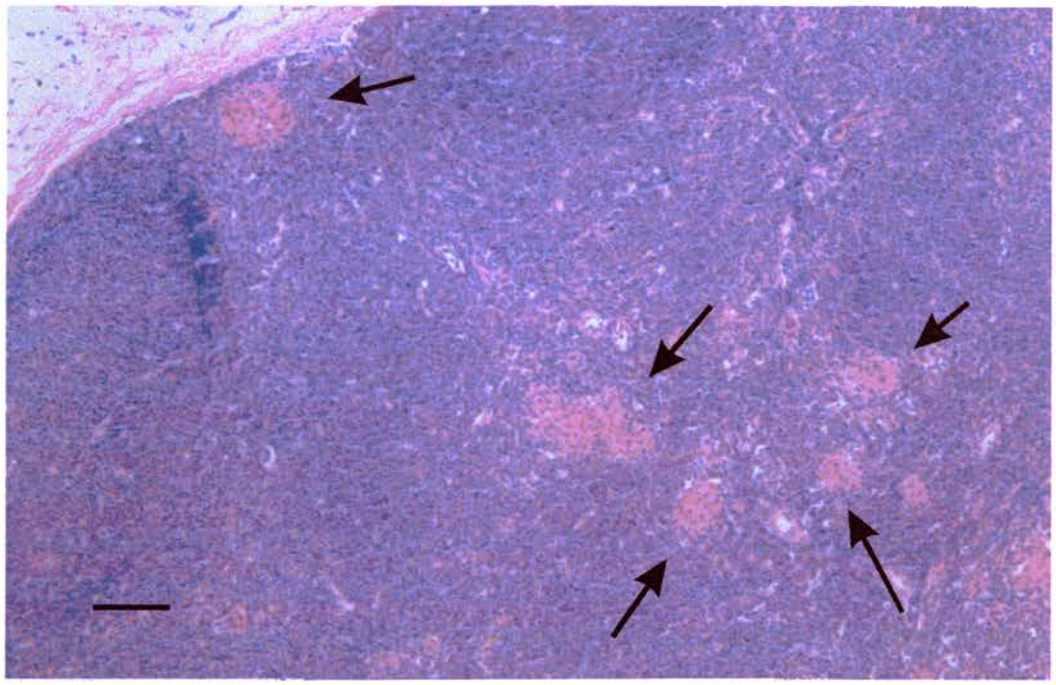


Plate 5,3 A section from the ICLN of calf 1543, experimentally infected with a bovine isolate of *M.a. paratuberculosis*. Discrete granulomata (arrows) are present scattered amongst the lymphocytes in the cortex of the lymph node. H&E Bar=100μm

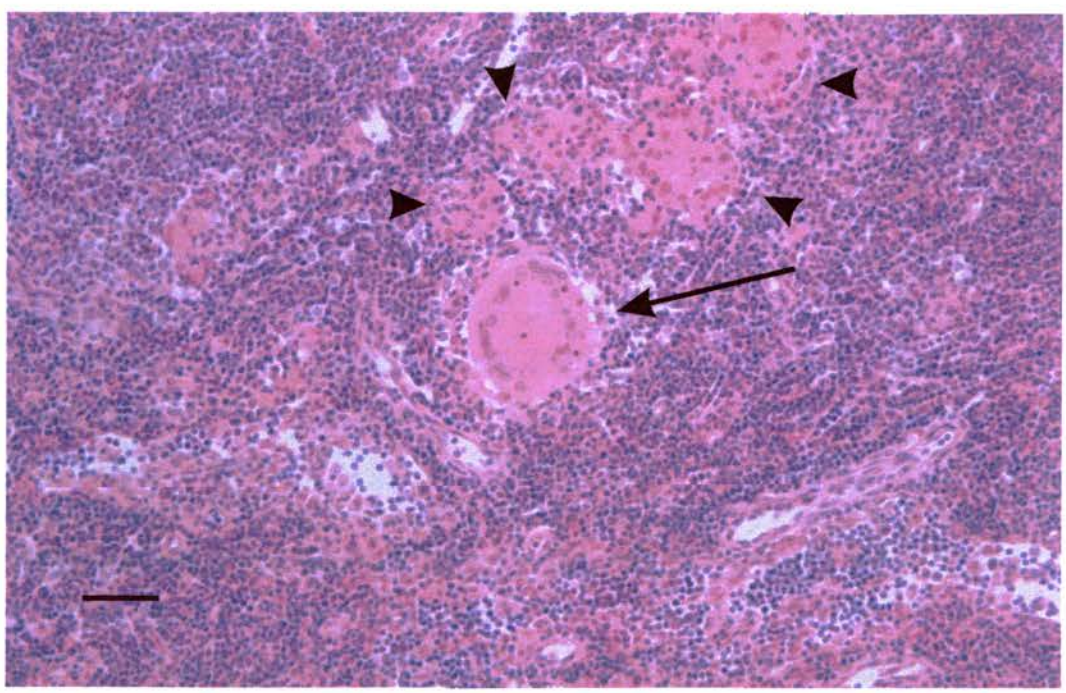


Plate 5,4 A section from the cortex of the ICLN of calf 1543, experimentally infected with the bovine isolate of *M.a. paratuberculosis*, showing a giant cell (arrow) and epithelioid cells (arrowheads) amongst the lymphocytes H&E Bar=50μm

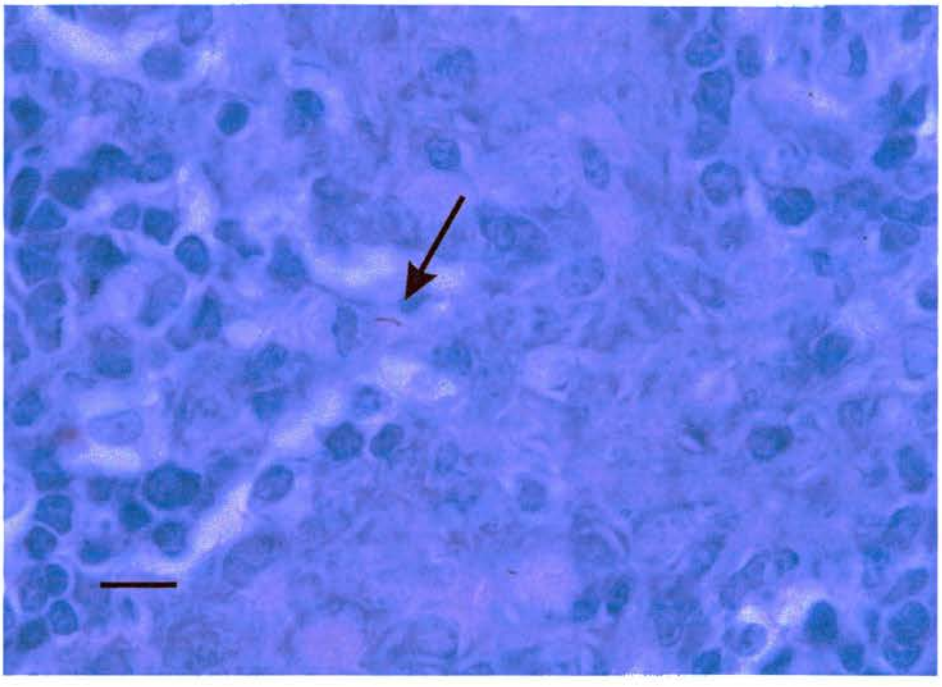


Plate 5,5 A section from a granuloma in the ICLN of calf 1545, inoculated with a bovine isolate of *M.a. paratuberculosis*. Note the AFB (arrow)
ZN Bar=10μm

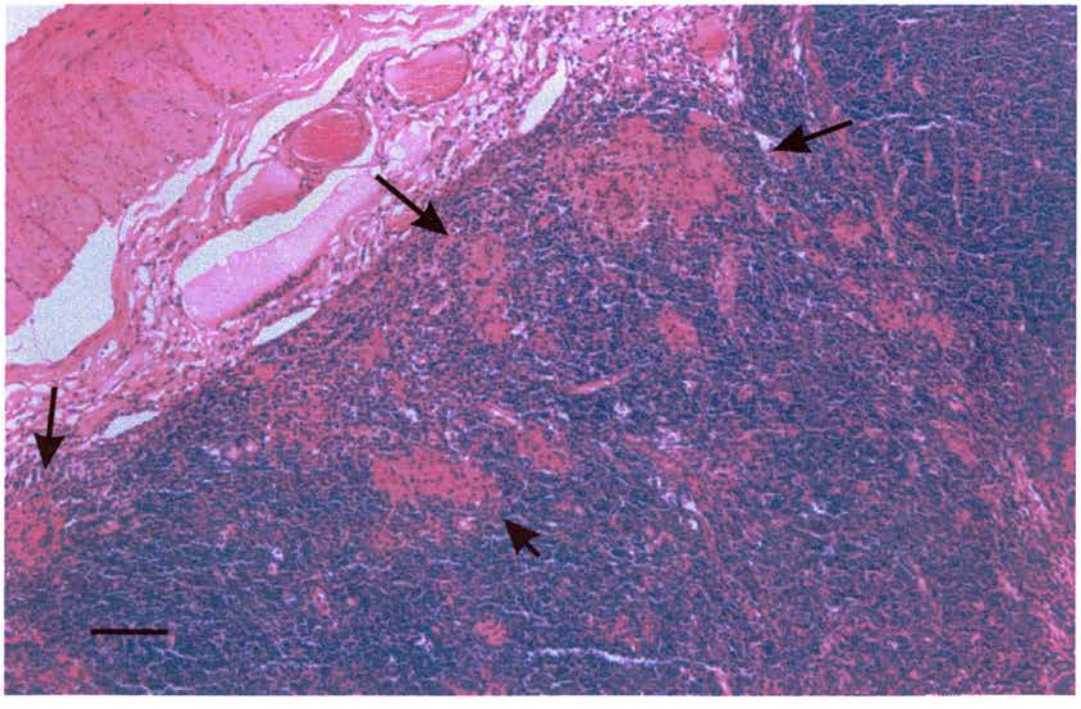


Plate 5,6 A section from the JPP of calf 1545, experimentally inoculated with a bovine isolate of *M.a. paratuberculosis*. Note the numerous granulomata present (arrows) at the base of the lymphoid tissue
H&E Bar=100μm

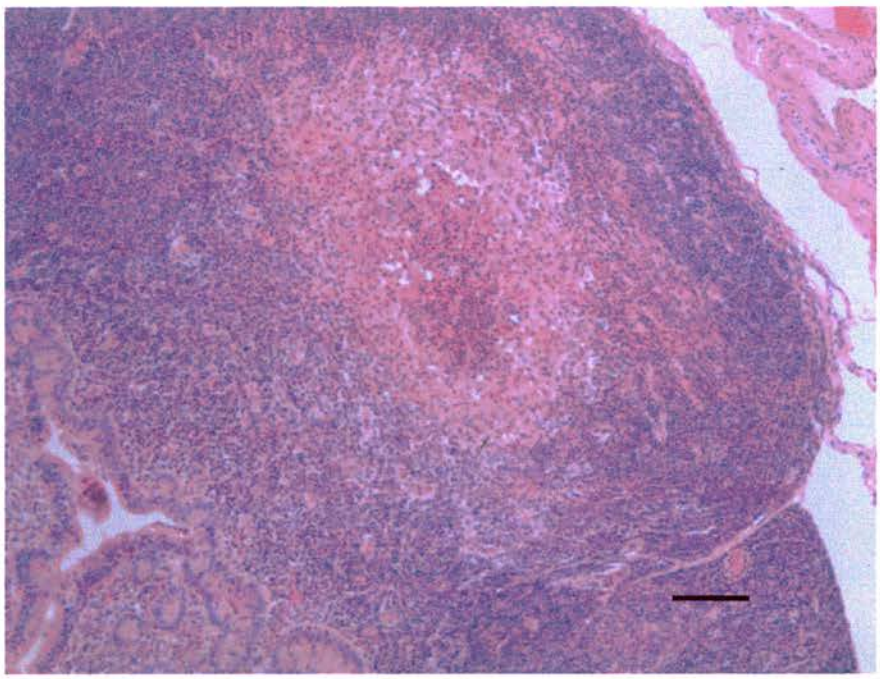


Plate 5,7 A section from the JPP of a calf from the control group. Note the large pyogranuloma, consisting of polymorphonuclear cells in the centre surounded by macrophages, present in the lymphoid follicle. No AFB were noted in serial ZN stained sections, therefore it was concluded that the pyogranuloma was related to coccidial infection H&E Bar=100μm

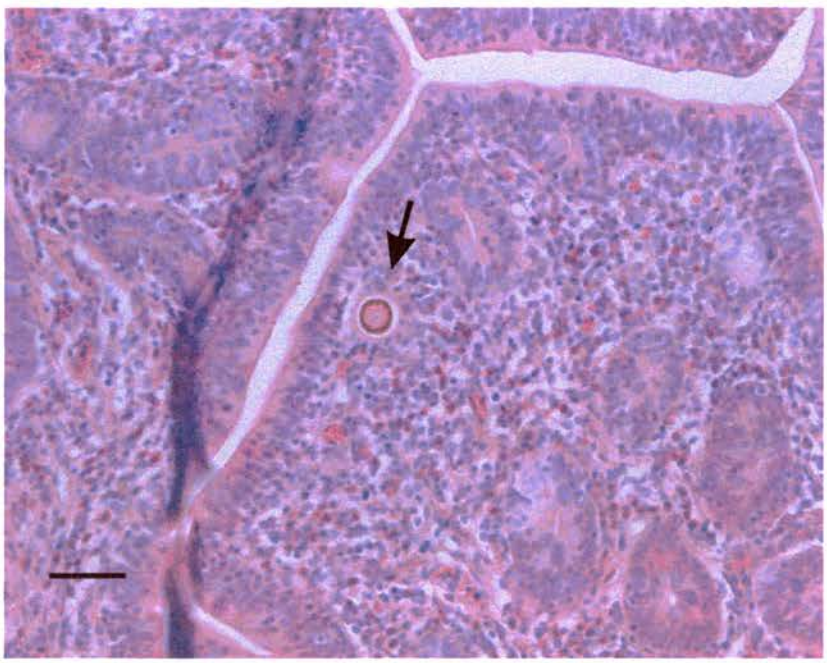


Plate 5,8 A section of villi from the jejunum of a calf with grade 1 coccidiosis. The only evidence of infection in this animal was the presence of one oocyst (arrow)
H&E Bar=50μm

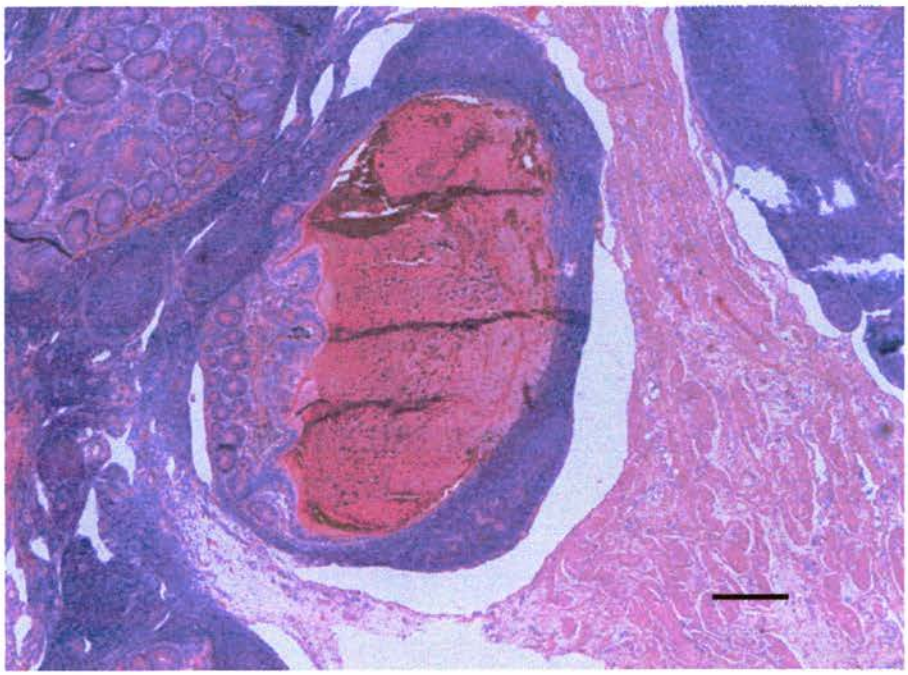


Plate 5,9 A large abscess in the connective tissue underlying the ICV in a calf inoculated with a rabbit isolate of *M.a. paratuberculosis*. It was concluded that this lesion related to coccidial infection. H&E Bar=250μm

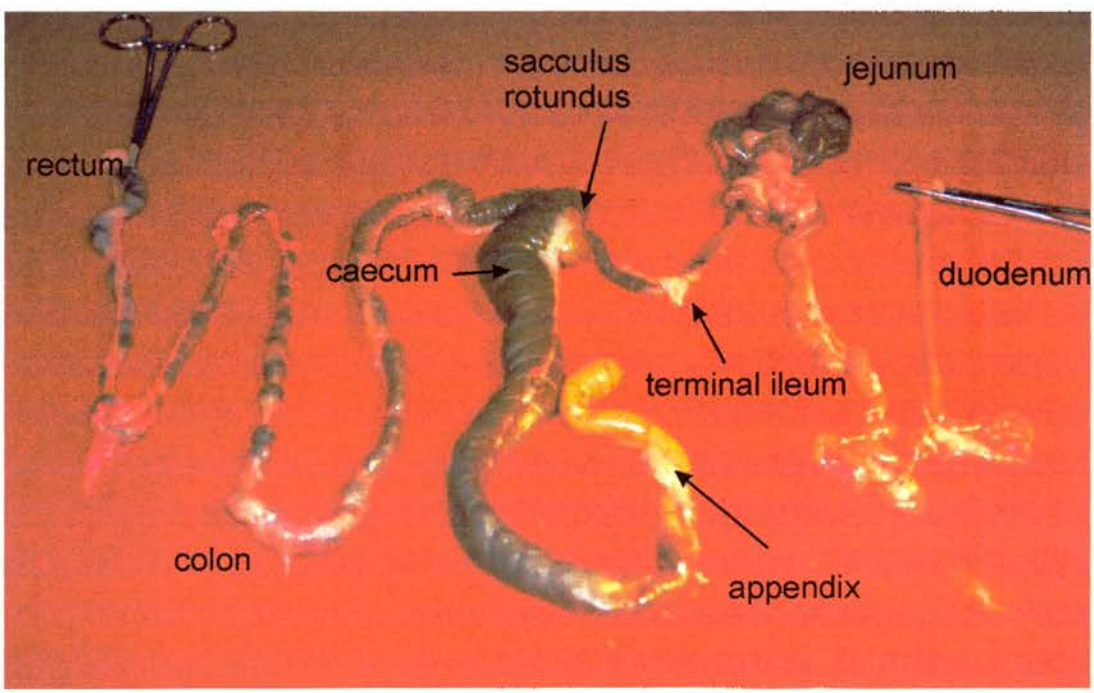


Plate 5,10 The gastointestinal system of a rabbit

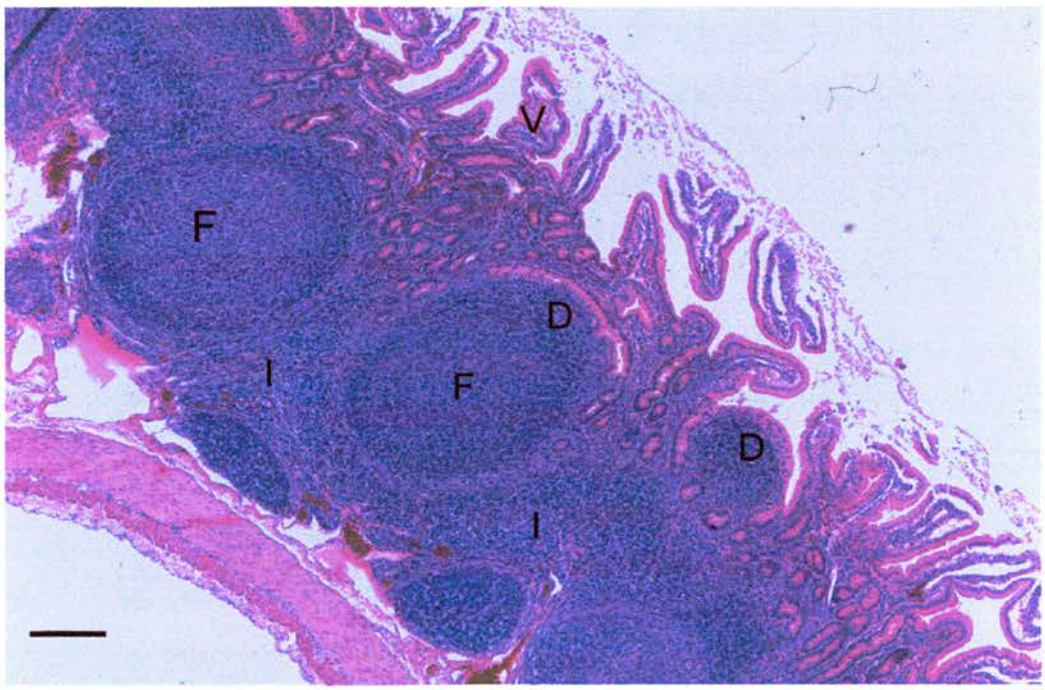


Plate 6,1 A section of normal JPP from a lamb, showing the four areas - the villous (V), dome (D), follicular (F) and interfollicular areas (I).
H&E Bar=250μm

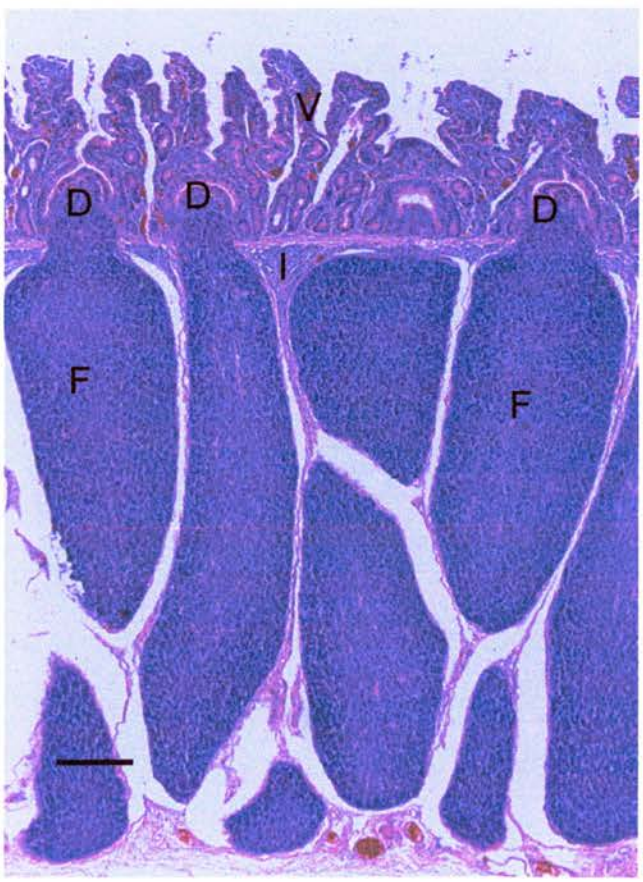


Plate 6,2 A section of normal IPP from a lamb, with the lymphoid follicles (F) below the villi (V) more encapsulated than the JPP seen in plate 6,1 above. The dome (D) and interfollicular areas (I) are also labelled.
H&E Bar=250μm

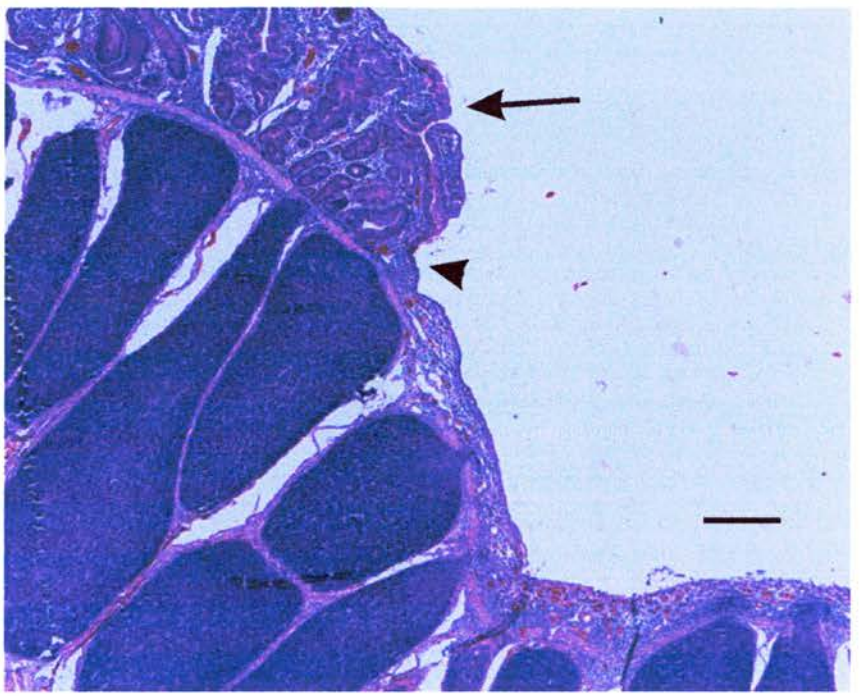


Plate 6,3 A section from the ileum of the one grossly abnormal lamb from experiment A, chapter 6. The underlying lymphoid tissue appears normal, and a zone of normal villi can be seen (arrow), however, there is a marked loss of normal villous architecture on the right of the section, with an abrupt demarkation between the two (arrowhead).
H&E Bar=250μm

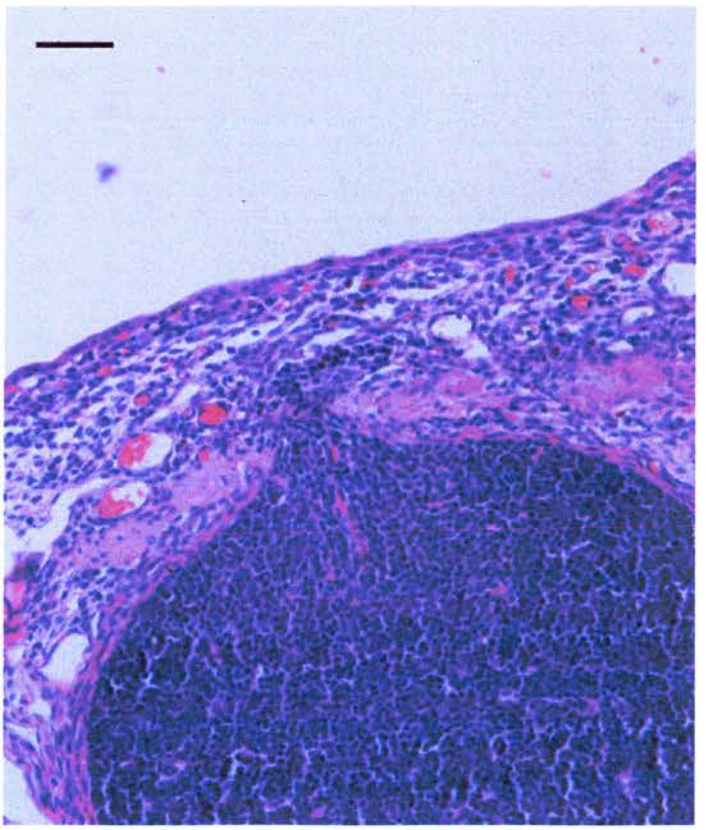


Plate 6,4 Higher magnification of the affected villous area from plate 6,3 above. There is complete flattening of villi, with a line of mildly attenuated epithelial cells present on the surface of the mucosa.
H&E Bar=50μm

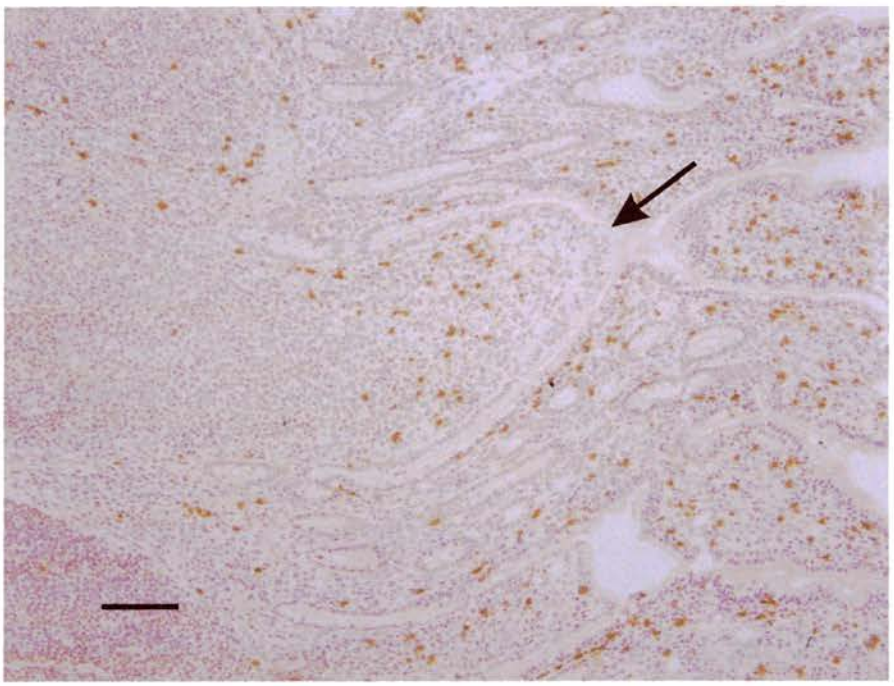


Plate 6,5 Immunohistochemical labelling of γδ T cells in the IPP of an inoculated lamb, showing positively labelled cells in the lamina propria and a dome area (arrow).
Bar=100μm

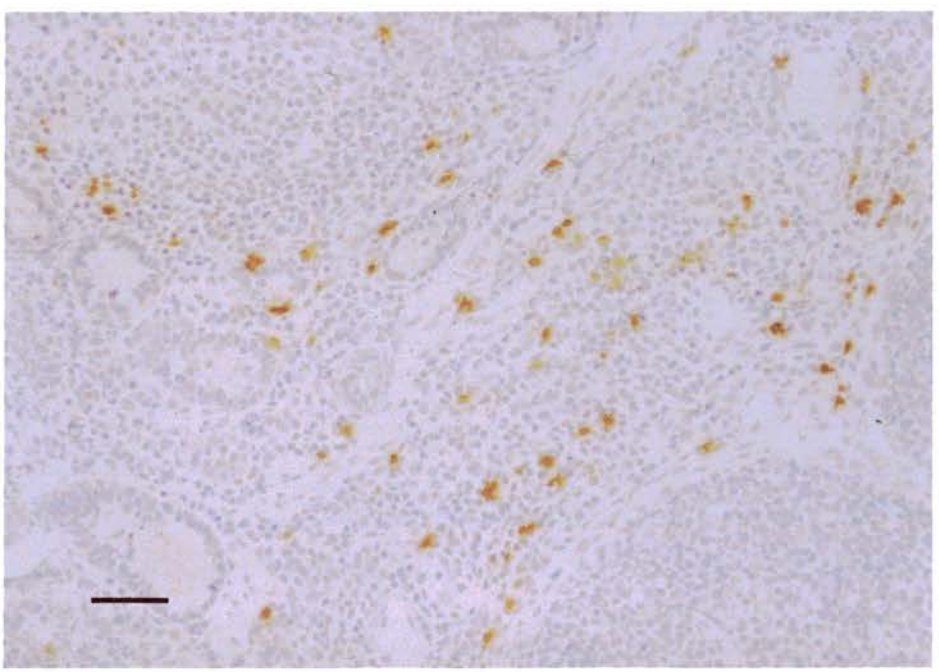


Plate 6,6 An immunolabelled section of IPP of an inoculated lamb, showing cells expressing CD1 molecules.
Bar=50μm

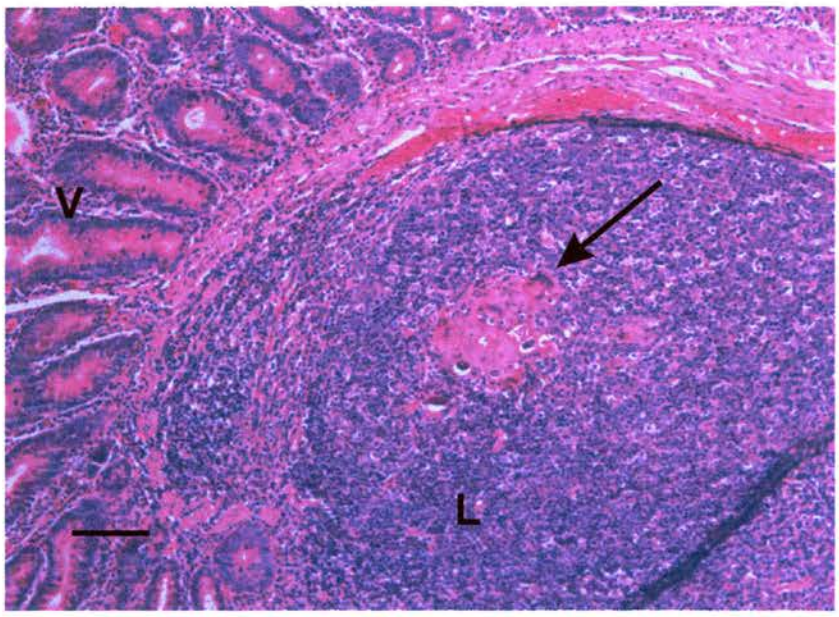


Plate 6,7 A section of lymphoid tissue (L) and overlying villi (V) from the JPP of a lamb experimentally inoculated with *M.a. paratuberculosis.* Note the granuloma present in the lymphoid follicle (arrow)
H&E Bar=100μm

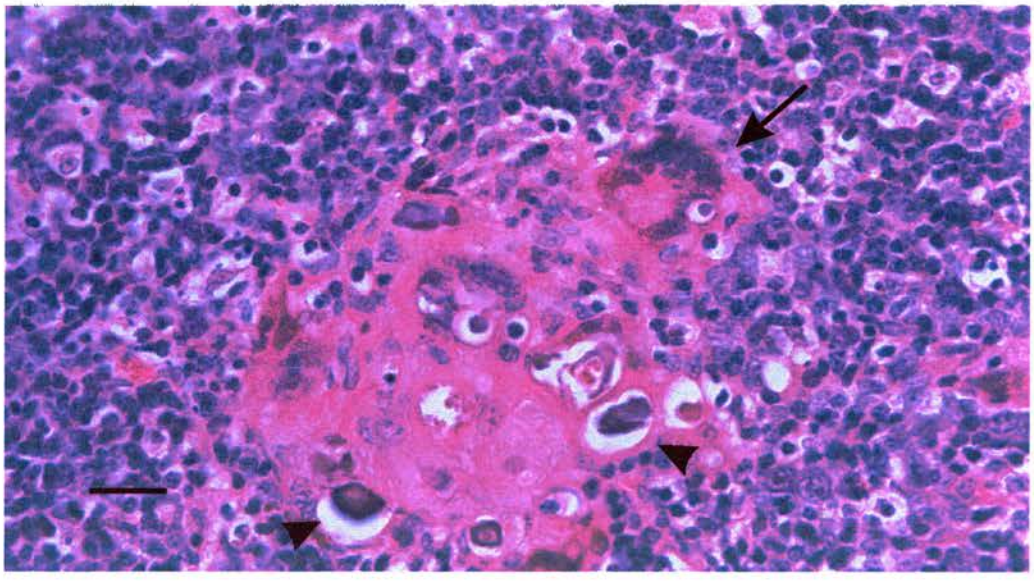


Plate 6,8 A higher magnification of the granuloma in plate 6,7, showing a giant cell (arrow) and degenerating cells (arrowheads)
H&E Bar=25μm

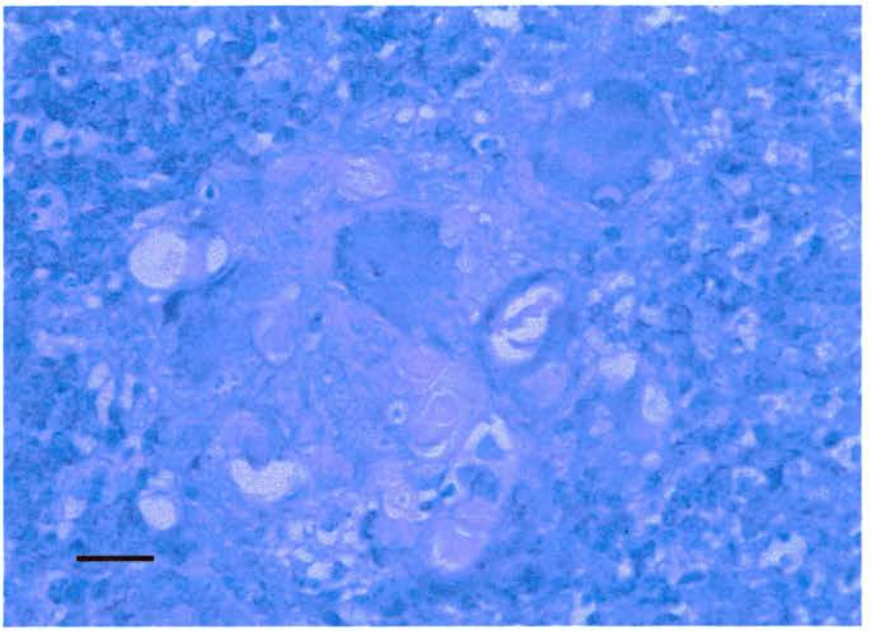


Plate 6,9 A serial section of plate 6,8, showing the absence of AFB in the granuloma
ZN Bar=25μm

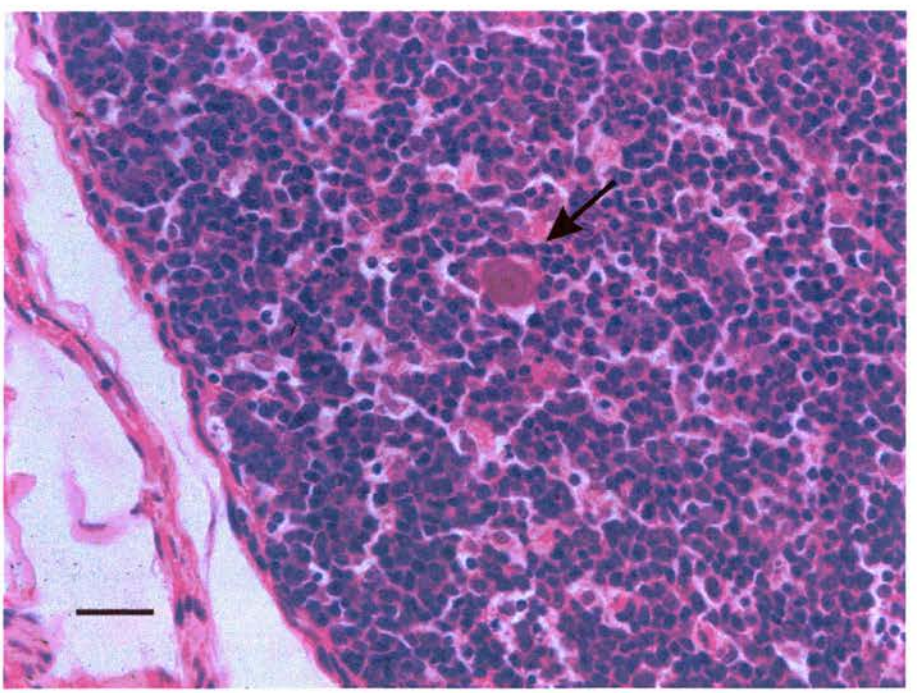


Plate 6,10 A section from the JPP of a lamb, with a degenerating cell present (arrow)
H&E Bar=50μm

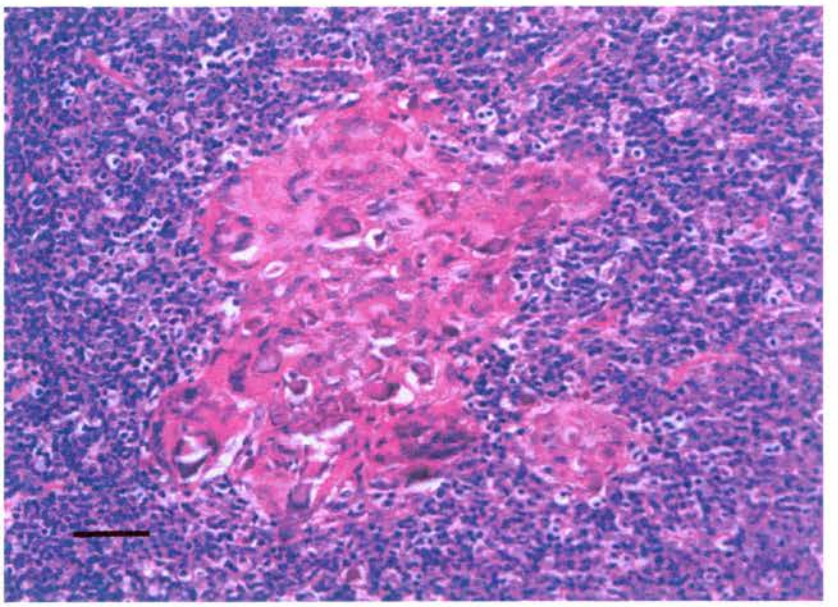


Plate 6,11 A section from the JPP of a lamb experimentally inoculated with *M. a. paratuberculosis,* showing a granuloma containing macrophages and numerous degenerating cells.
H&E Bar=50μm

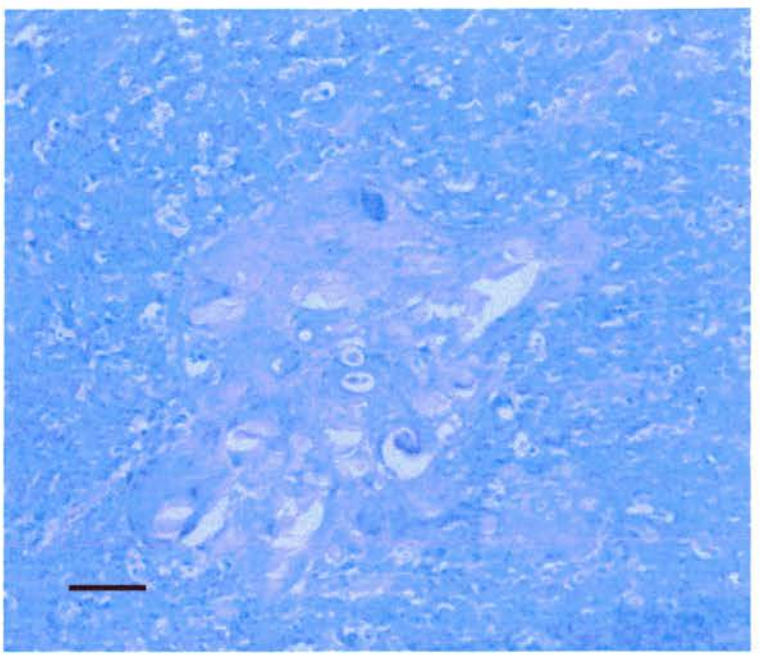


Plate 6,12 A serial section of plate 6,11 above, revealing no AFB in the granuloma
ZN Bar=25μm

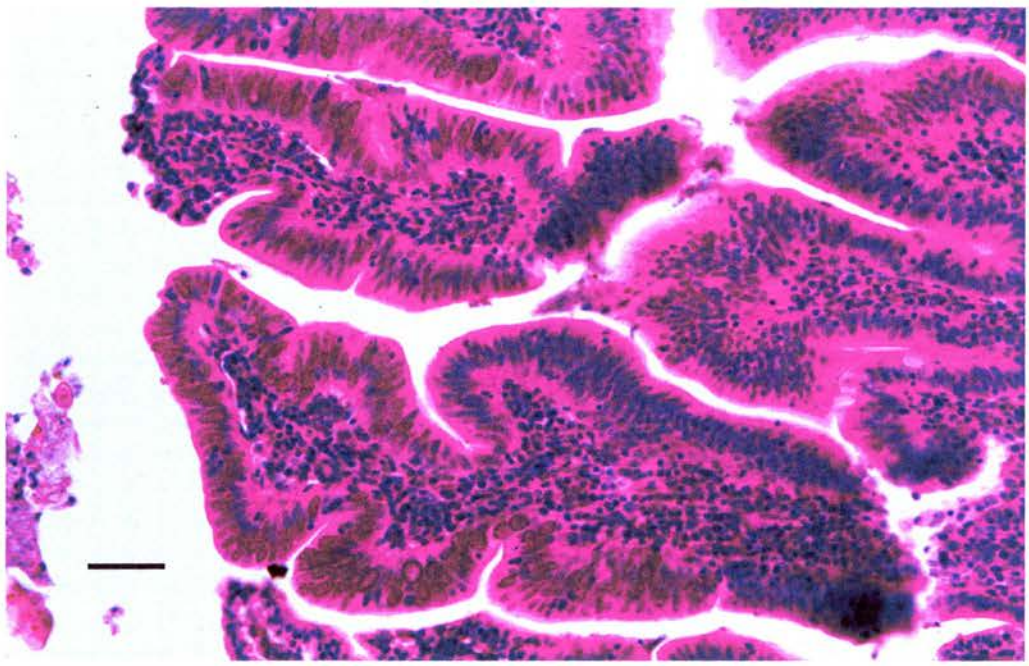


Plate 6,13 Coccidial oocysts present amongst the epithelial cells of the ileum in a lamb. Note the lack of accompanying inflammatory reaction
H&E Bar=50μm